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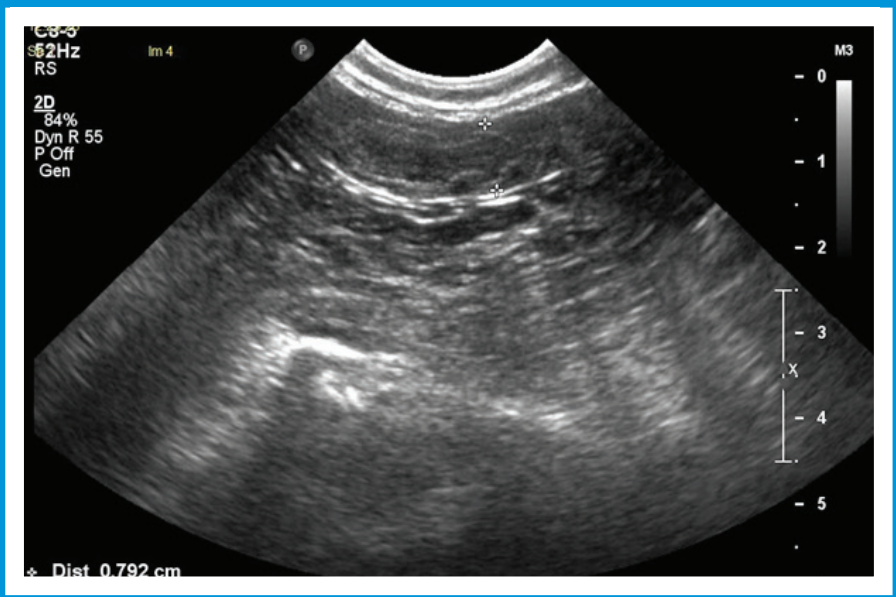
SLOVENSKI VETERINARSKI ZBORNIK



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Review Article

EFFECTS OF SYNTHETIC AND ORGANIC ACARICIDES ON HONEY BEE HEALTH: A REVIEW

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Abstract: The honey bee is a crucial pollinator of agricultural crops and also an economically important producer of commodities such as honey and beeswax that find diverse uses in the food industry, cosmetics and medicine. At present, the ectoparasitic mite *Varroa destructor* is viewed as the most damaging pest of the honey bee worldwide. Without treatment, colonies generally collapse within a few years. To keep the population of the Varroa mites low, beekeepers relay on the use of synthetic and organic acaricides, the most popular commercially available ones include amitraz, coumaphos, flumethrin, fluvalinate, formic acid, oxalic acid and thymol. These conventional acaricides are cheap and easy to apply, but prolonged use causes Varroa mites to rapidly develop resistance and bee products can become contaminated. Residues of acaricides are present in high concentrations throughout the hive and bees are exposed to them all year around. The present review summarises the current knowledge of the deleterious effects of conventional acaricides on honey bee health. Numerous commercially available acaricides and their active substances have been shown to have negative effects on honey bee brood development, queen and drone reproductive health, learning, longevity and colony strength. Acaricides do not only act alone, but also in synergic combinations to affect bee health. Since some drugs cause substantial weakening of bee colonies, they can make them more susceptible to other diseases such as nosematosis or to extreme climatic events. As wax combs are contaminated with high concentrations of acaricide residues and Varroa mites are chronically exposed to them, the parasite may develop resistance faster. In combination with other stressors, acaricides could be a contributing factor to colony collapses.

Key words: synthetic acaricides; varroacides; honey bee; *Apis mellifera*; synergy; organic beekeeping;

Introduction

The honey bee (*Apis mellifera* L. 1758) ranks among the most economically important pollinators in the world, providing key ecosystem services in both artificial and natural landscapes (1). It has been estimated that the worldwide value of pollination is about €153 billion annually (2), making honey bees key contributors to global economy. Also important is the role of honey bees in collecting and manufacturing products including honey, beeswax, propolis, royal jelly or bee venom that today find wide uses as foods, natural medicines and cosmetics. Beekeeping is the source of income for many around the world and a key poverty alleviation measure (3). However, there are a number of pests and parasites of the honey bee that require control. As of present, the Varroa mite (*Varroa destructor*) is considered to be the most damaging parasite of the honey bee worldwide (4). Without intervention most, but not all, colonies collapse within several years. Today, Varroa treatments consist primarily of chemotherapy (5).

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In Europe, the most frequently used chemicals to control *V. destructor* are synthetic compounds such as coumaphos (active ingredient in medicaments such as Check-Mite™ or Perizin®), fluvalinate (Apistan®, Gabon®), flumethrin (Bayvarol®) and amitraz (Apivar®, Varidol®) (6). In attempt to find more "natural" cures to varroasis, oxalic acid, formic acid (Formidol®) and thymol (Thymovar®) have been introduced and are becoming increasingly popular among organic beekeepers (7). Organic acids and thymol occur naturally in low concentrations in honey, thereby their organic reputation. An overview of the most popular acaricidal chemicals used globally is given in Tab. 1. Today, many of the medicaments originally developed to control *V. destructor* are also used to control other widespread bee parasites such as *Tropilaelaps* mites or bee lice (*Braula coeca*) (8, 9).

bee products (13). Residues of some acaricides have been shown to be very persistent and present a concern for human health (14). Equally importantly, the residues of acaricides can also have serious consequences for the health of the colony.

As of present, much research concentrated on the quantification of acaricide residues in hive products but relatively few studies addressed the impact of residues on honey bee health. This question is also highly controversial given the financial interest of a number of bee research organisations in manufacturing and selling bee medicaments. This review presents a compilation of the published effects of common commercially available synthetic and organic acaricides on honey bee health.

A review of the negative side effects of acaricides is listed separately for every active ingredient

Table 1: Examples of popular acaricides used across the world (10)

Compound	Examples of Commercial Products	Residues	Resistance
Amitraz	Apivar® Varidol®	wax, pollen, honey	described
Coumaphos	Checkmite™ Perizin®	wax, pollen, honey	described
Flumethrin	Bayvarol®	wax, pollen, honey	described
Fluvalinate	Apistan®	wax, pollen, honey	described
Formic acid	MAQS®	not known	not known
Oxalic acid	Bienenwohl® Oxuvar®	not known	not known
Thymol	Apilife VAR® Apiguard® Thymovar®	not known	not known

No pesticide used in honey bee medicine is 100% efficient. "Easy to use" drugs were initially seen as a simple, cheap and fast solution to honey bee disease problems, but their widespread use and loose legislation control in some countries resulted into Varroa populations rapidly developing resistance. As such, beekeepers are made reliant on more and more chemical products (11).

Following the application of these medicaments, residues can be detected throughout the beehive in products such as royal jelly but also in adult bees and brood (12). Acaricide residues constitute a significant portion of chemical contaminants of

in Tables 2-7. The impacts of these chemicals on honey bee colony health are discussed systematically in the sections below.

Negative Effects of Acaricides on Honey Bees

Effects on Adult Worker Bees

A mounting body of research (reviewed in Tab. 2-7) agrees that among others, exposure of bees to some acaricides at recommended doses can lead to high bee mortality and shortened lifespan. But

what affects the resistance of individual bees to acaricide intoxication? There seem to be several factors. Coumaphos and fluvalinate are more toxic to older bees then young bees (15, 16 cited in 17). Workers that were subjected to less stress appear to be more resistant to fluvalinate and coumaphos poisoning (17, 18). Bee mortality can also increase with higher outdoor temperature. Thymol is practically harmless at outdoor temperatures ranging from 5°C to 9°C (19), but was linked with high bee mortality at temperatures above 27°C (20, 21). The tolerability of the oxalic acid treatment depends strongly on the method of administration used. Colonies treated with the trickling method had a significantly higher adult bee mortality then those treated with the vaporizer method (22). Quite interestingly, it has been shown that queen bees can tolerate higher doses of acaricides than workers, suggesting that the physiological differences between the two can affect pesticide sensitivity (23). Other factors that may affect the mortality of workers that contacted acaricides include their pathogen load (24), mobility (25) and the acaricide dose received (26, 27). On the other hand, the strength of the colony seems to not affect the resistance of bees to acaricide application (28).

Acaricide exposure also affects the bee´s behaviour. Bees fed with coumaphos had reduced trophallaxis which could severely affect the food transfer and energetic distribution of the whole colony (29). Organic acaricides such as formic acid or thymol are irritant to bees and cause an increase in fanning (30 cited in 31, 32, 33), probably to rid their hive of unwanted acaricides vapours.

Negative effects of acaricides on bees also include impaired metabolism. Bolli et al. showed that under laboratory conditions, high concentrations of formic acid in the air inhibit oxygen intake in young adult bees (34). Application of amitraz and flumethrin leads to a reduction of proteins, carbohydrates and lipids in the hemolymph of 0-, 7- and 21-day old worker bees (35). Adult bees as well as bee brood from colonies treated with amitraz (Apivar®), flumethrin (Bayvarol®) and thymol (Apiguard®) had lower levels of Glutathione S-Transferase activity (36).

Some acaricides have been shown to affect the physiology of adult worker bees. Formic acid treatment had a significant effect on the number of sensilla found in the bee's antenna. Non-significant differences were recorded for the mean length and surface area of the sensilla (37). Topically administrated oxalic acid is known to penetrate keratin and can subsequently be detected in the bee´s internal organs including the digestive tract, rectum and heamolymph (38), which it can damage and cause elevated mortality (39).

Table 2: The effects of amitraz on honey bees

Formulation	Exposure	Effects	Reference
Apivar®	2 strips per hive, 6 weeks	↓ proteins, ↓ carbohydrates, ↓ lipids in haemolymph of adults	(31)
Taktic®	aerosol	associated with weakening of treated colonies	(40)
in larval diet	25-440 ppb in larval diet	↓ chance of pupation ↓ chance of survival to adulthood ↓ defecation	(41)
	200 ppm	affects expression of some proteins	(42)
topical application to adults, 24-hour exposure	283 ppm per treatment group	↓ glucose dehydrogenase expression may compromise cellular immunity	(43)
topical application to adult queen bees	1.0µg/µl in acetone	↓ brood survival	(4)
		shortly after application breaks down into 2,4-dimethylaniline which is very persistent, mutagenic, oncogenic and genotoxic	(44, 45-48)

Table 3: The effects of coumaphos on honey bees

Formulation	Exposure	Effects	Reference
Check Mite+®	two strips per colony	↓ sperm viability in drones ↓ sperm numbers in drones ↓ viability of stored sperm queen bees fail to develop	(49, 50)
		in extreme cases, acute colony positioning (abnormal bee behaviour, loss of about 2/3 of the adult population)	(18)
		↑ pkac gene (detoxification) ↓ CYP306 gene (detoxification) ↓ VGMC gene (development) ↓ DSC37 gene (immunity)	(51)
		alters bee gut microbiome	(52)
		↑ emergency queen cell construction ↑ adult bee mortality ↓ brood survivorship	(53)
		↓ queen acceptance ↓ queen mating success ↓ drone production	(54)
Perizin®	10 µl solution	↑ volume of haemolymph	(15)
	oral administration to adult worker bees	3-day old worker bees are three-fold less susceptible to coumaphos then 8- and 13-day old bees	
	colonies treated according to manufacturer´s instructions	↑ bee mortality if colonies were exposed simultaneously to some organophosporus pesticides (parathionethyl, dimethoate, dialifos)	(55)
	20ml per colony	alters expression of immunoregulatory genes	(56)
10% coumaphos strips	one to four strips per colony	queen cells torn down queen bees fail to develop	(57, 58)
	¼ to ½ strips per colony	↓ queen weight ↓ ovary weight ↓ number of sperm	
beeswax cups treated with coumaphos	1 to 100 mg/kg coumaphos in beeswax	↓ egg laying in queens	(59, 60, 61)
	10 to 100 mg/kg coumaphos in beeswax	↑queen rejection ↓ queen pupal weight ↓ queen adult weight skews the relationship between queen weight and spermatheca size probably longer queen development time	
	100 to 1000 mg/kg coumaphos in beeswax	↑ queen rejection ↓ queen cell acceptance ↓ queen pupal weight ↓ queen adult weight queens fail to develop probably longer queen development time	

caged bees fed coumaphos	2 µg of coumaphos per cage	↑ worker mortality	(29)
	5µg of coumaphos per cage	↑ worker mortality ↓ trophallaxis	
	100 ppm per treatment group, dissolved in sucrose solution	alters the expression of some genes related to detoxification, behavioral maturation, immunity	(62)
	in queen candy	↑ larvae mortality in combination with fluvalinate, ↓ intestinal stem cell proliferation	(63, 64)
coumaphos in acetone	1-3µg dissolved in 1 µl of acetone, topical application to adult worker bees	↑ adult worker mortality toxicity of coumaphos ↑ by up to 3.4-fold when fluvalinate is present	(65, 66)
	5ppm coumaphos in acetone, 2µl topical application to queens	alters the expression of P450 subfamily genes, antioxidant, immunity and development genes	(67)
acaricide-laden combs from a beekeeping operation	bees reared in combs containing 281 to 6311000 ng/g coumaphos	↑ *Nosema ceranae* infection rate ↑ brood mortality ↓ lower longevity delayed larval development delayed adult emergence	(68, 69, 70)
in larval diet	50 ppm	affects expression of some proteins	(42)
topical application to adults, 24-hour exposure	751 ppm per treatment group	↓ hymenoptaecin and ↓ abaecin expression may compromise cellular immunity	(43)

Table 4: The effects of flumethrin on honey bees

Formulation	Exposure	Effects	Reference
Bayvarol®	4 strips per colony for six weeks	↓ proteins, ↓ carbohydrates, ↓ lipids in haemolymph of adults	(35)
	12 strips per colony	↑ glutathione S-transferase activity (biomarker of toxic stress) in larvae, pupae and nurse bees ↓ lower protein content in treated bees	(71)
topical application to adults	751 ppm per treatment group, 17-hour exposure	↑ hymenoptaecin expression may compromise cellular immunity	(43)

Table 5: The effects of fluvalinate on honey bees

Formulation	Exposure	Effects	Reference
Apistan®	two strips per colony	↓ drones survive the first day of their adult life ↓ drone longevity ↓ drone weight ↓ drone mucus gland weight	(72)
	two strips per colony for 24-28 days three times during the season	suspected to negatively affect queen bee survivorship	(73)
	two strips per colony for four weeks	↑ worker mortality ↑ glutathione S-transferases activity ↓acetylcholinesterase activity	(74, 75)

Table 5: continuation

Formulation	Exposure	Effects	Reference
Apistan®		↓ sperm numbers in drones	(54)
	two strips per colony for six weeks	alters bee gut microbiome	(52)
	two strips per colony for 42 days	↑ emergency queen cell construction ↓ brood survivorship	(53)
	according to manufacturer's instructions, for three and seven days	in combination with coumaphos, ↓ intestinal stem cell proliferation	(64)
Apistan Queen Tab®	caged bees exposed to one strip for five days	↑ worker bee mortality	(24)
Fluwarol™	two strips for six months	associated with weaker colony development, drone laying and queen death contaminated combs probably induce laying of unfertilized eggs by queens	(76)
10% fluvalinate strips	eight strips per colony for 10 days	↓ queen weight	(57, 58)
1% fluvalinate strips	caged bees exposed for 3 days	↑ queen supersedure ↑ worker bee mortality	(17)
	caged bees exposed for 7 days	↑ queen mortality	
fluvalinate in acetone	10 mg fluvalinate in acetone per dish, caged workers exposed for 24-hours by contact method	↓ rate of odour learning	(77)
	dissolved in 1-1.25 µl of acetone, topical application to adult workers	↑ adult worker mortality toxicity of fluvalinate ↑ if other pesticides are present	(65, 66)
	0.125µg-1.25µg, dermal application	affects learning, memory and responsiveness to sucrose	(78)
	0.125µg-1.25µg, oral application		
	0.3µg-3µg topical application	↓ worker mobility	(79)
beeswax cups treated with fluvalinate	100-1000 mg/kg coumaphos in beeswax	↑ queen rejection ↓ queen production probably longer queen development time	(60)
caged bees fed fluvalinate	100 ppm per treatment group, dissolved in sucrose solution	alters the expression of some genes related to detoxification, behavioral maturation, immunity	(62)
	in queen candy	↑ larvae mortality	(63)
in larval diet	100 ppm	affects expression of some proteins	(42)

acaricide-laden combs from a beekeeping operation	bees reared in combs containing 68000-104000 ng/g fluvalinate	↑ brood mortality ↑ *Nosema ceranae* infection rate ↓ lower longevity delayed larval development delayed adult emergence	(68, 70)
		suspected to ↑ larvae mortality	(80)

Table 6: The effects of formic acid on honey bees

Formulation	Exposure	Effects	Reference
Apivarol®	three fumigations one tablet each, then 40 ml of 60% formic acid per colony in evaporators	↓ pH of honey stores throughout the following season	(81)
Bee Var	one gel packet per hive, two treatments in two week intervals	interrupts brood rearing	(82)
Miteaway™	according to manufacturer´s instructions	↑ pkac gene (detoxification)	(51)
Varterminator®	6% formic acid gel for 20 days	↑ egg mortality by about 77%	(83)
85% formic acid	10 ml per colony for 30 days	↑ heat shock proteins in bee brains, a molecular indicator of stress	(84)
	in deionized water, applied by evaporation	cell death in gut	(85)
65% formic acid	22g of formic acid per absorbent pad, colonies treated four times at four day intervals	application can drive bees out of their hive and irritate them	(30)
	40ml per absorbent pad three times in weekly intervals	application can drive bees out of their hive, ↑ fanning behaviour and irritate them	(32)
	200ml per Ziploc bag for 40 days	↓ worker population ↓ worker brood area	(86)
	200ml per absorbent pad for 6 weeks	↓ brood survival	(87)
	cotton strips saturated with formic acid and black cumin oil suspension, replaced weekly for 32 weeks	negative effects on sensory organs in the antennae	(37)
	one gel pack per colony for 21 days	↓ adult drone survival delays drone production drone eggs removed from combs	(88)

Table 6: continuation

Formulation	Exposure	Effects	Reference
60% formic acid	20-40ml per colony	↓ larval feeding	
	200ml per colony for five weeks	↑ H+ and H- natural protease inhibitor activities in workers ↑ H+ protease activities in pupae ↓ antifungal and antibacterial activities of adult and larvae worker´s cuticle ↓H+ protease activities in workers and larvae ↓ H+ and H- natural protease inhibitor activities in larvae and pupae activates thiolic proteases	(89)
30% formic acid	10 ml per colony for 30 days	↑ heat shock proteins in bee brains, a molecular indicator of stress	(84)
40ml in absorbent pad	five applications in 4-day intervals	↓ area of sealed brood bees clear brood cells close to the absorbent pads associated with queen loss	(90)
formic acid odour	applied by the fumigation method	induces aversion in adult worker bees	(91)
fumigation	60 ppm formic acid for 9 days per overwintering room	↑ worker bee mortality associated with queen losses	(92)
	45 ppm formic acid for 11 days per overwintering room	associated with queen losses	

Table 7: The effects of oxalic acid on honey bees

Formulation	Exposure	Effects	Reference
Api-Bioxal®	35 g of Api-Bioxal® dissolved in 500 ml	↑ adult bee mortality	(22)
dissolved in sucrose solution	dissolved in 50% sucrose solution, applied twice by trickling to hives	↑ brood removal ↓ amount of brood	(93)
	50 mL of 3% oxalic acid in 32% sucrose solution (w/w), applied once	↑ capped brood removal	(94)
	50 ml of 1.5g oxalic acid in sucrose solution, applied by trickling twice in a 3-week interval	↑ bee mortality	(39)
	2% solution of oxalic acid in an aqueous 50% sucrose solution by oral administration	can penetrate keratin and reach the bee´s internal organs	(38)

Formulation	Exposure	Effects	Reference
dissolved in sucrose solution	30-45g of oxalic acid per 1l of sucrose solution, trickling 5-6ml per bee space	higher bee mortality with increasing doses	(95)
	3.2% solution of oxalic acid in 50% sucrose solution by trickling 5ml per bee space	↓ pH of honey stores throughout the following season ↑ heat shock proteins in bee brains, a molecular indicator of stress	(81, 84)
	4.2% solution of oxalic acid in 50% sucrose solution by trickling 5ml per bee space	↓ colony strength	(96)
in aqueous solution	submersing bees in 1%-2% oxalic acid in aqueous solution	↑ adult bee mortality	(27, 97)
	spraying bees with 1%-1.5% oxalic acid in aqueous solution, 25ml per frame	↑ adult bee mortality colony weakening	
	spraying bees with 0.56%-2.25% oxalic acid in aqueous solution, 50 ml per frame	↑ colony mortality ↑ adult bee mortality ↓ brood	(28)
	spraying bees with 3% oxalic acid in aqueous solution, 8ml per frame	↓ brood associated with queen death	(98)
	10% (w/w) oxalic acid in water and 1% Tween® 20, 6 µL by topical application per bee	permanent damage to the bee's digestive and excretory organs	(39)
	10%-20% oxalic acid in water, 6 µL by topical application per bee	can penetrate keratin and reach the bee´s internal organs	(38)
topical application to larvae	0.121 mg per larva	cell death in gut and salivary glands	(85, 99)
sublimation	3.6g per hive	↑ heat shock proteins in bee brains, a molecular indicator of stress	(84)

Table 8: The effects of thymol on honey bees

Formulation	Exposure	Effects	Reference
Apilife VAR®	two wafers per colony applied three times in 51 days	↓ amount of brood	(100)
	applied for 42 days, replaced at 21 days	slows down colony development (measured by adult bee population, brood, stored pollen) associated with ↓ brood	(101)
	one tablet per colony	↓ food intake in treated colonies ↓ sealed brood area bees empty cells next to the tablets bees remove most of the product in 10 days	(102, 103)

Table 8: continuation

Formulation	Exposure	Effects	Reference
Apilife VAR®	one tablet per colony, replaced in 10-day intervals	↓ sperm numbers in drones ↓ percentage of live spermatozoa in queens	(49)
	one tablet per colony, three applications in 7-day intervals	↓ phototaxis	(104)
Apiguard®	one gel per colony	↑ larval and pupal mortality ↓ sealed brood area bees remove most of the product in 10 days	(103, 67)
	one gel per arena	avoided by older workers contact via bee antennae induces fanning behaviour	(33)
	according to manufacturer´s instructions	↑ CYP6a514 gene (detoxification) ↑ pkar gene (detoxification) ↑ pkac gene (detoxification) ↓ CYP306 gene (detoxification) ↓ VGMC gene (development) ↓ DSC37gene (immunogen) ↓ BASKgene (immunogen)	(51)
	20 days	when applied in conjunction with queen caging ↓ the colony population	(105)
Ecostop®	30 days	↑ heat shock proteins in bee brains, a molecular indicator of stress	(84)
Thymix	20-40ml per colony	brood damage	(106)
Thymovar®		if the temperature exceeds 30°C brood dies off	(Thymovar® manufacturer instructions)
thymol in acetone	3-10 µg by topical application per bee	probably ↓ drone flight activity	(107)
	50 mg of thymol per kg of food by oral administration to larvae	altered vitellogenin expression patterns	(108)
thymol in acetone	500-100 mg of thymol per kg of food by oral administration to larvae	↓ larval survival ↓ larval weight	(108)
thymol-oil spray (4.8 thymol mol/l in 20% canola oil)	400 ml sprayed per colony	associated with queen deaths	(109)
thymol in vermiculture (3.6g per block)	one block per colony replaced every four days for 24 days	↑ adult bee population	
thymol powder	0.5g per comb	application disturbed bees	(19)

Effects on Queen Bees and Drones

In recent years, beekeepers have reported mounting problems with queen filatures. As a result, a large body of research investigated the effects of acaricides on the reproductive biology of queen bees as well as drones. In general, insecticides can lower the percentage of matings, female fecundity and reduce egg hatch in arthropods (110). In *A. mellifera*, where a single queen is the only reproductive individual, this could have dramatic effects. Acaricides can lower queen bee reproductive potential throughout hindering comb construction (and thus giving the queen bees less space to lay eggs), reducing the egg production, the ability to requeen and supersedure queens secessfully (111).

Several studies were devised to test whether queen bees reared in wax cups contaminated with acaricides are negatively affected. Indeed, it was shown that coumaphos treatment drastically decreases the acceptance rate of grafted queen cells (54). In addition, queen bees reared in queen cups containing 1000mg/kg of coumaphos failed to develop. A concentration of 100mg/kg causes over 50% queen rejection; the surviving queens weigh significantly less the untreated controls (59). Queen bees exposed to coumaphos or fluvalinate show high mortality as well as lower ovary weight, lower sperm numbers, lower body mass, physical abnormalities, atypical behaviour and other characteristics that make them unsuitable for commercial use (49, 57, 58, 60, 61, 112). Fluvalinate contamination of the queen cells also reduced the number of queens reared (60). Collins et al. further underlined that the adverse effects of acaricide wax contamination on queen rearing may be further deepened by stressful conditions such as excessively hot, cool, dry or humid weather, high initial *V. destructor* levels or migratory beekeeping practices (60). Under filed conditions, the pesticides beekeepers apply in their hives can combine and their synergic interactions can amplify the negative effects on honey bee reproductive characteristics. Honey bee queens raised in wax cups containing high concentrations of both coumaphos and fluvalinate, at 94 ppm and 204 ppm respectively, had significantly lower sperm counts and sperm viability (112).

Haarmann et al. proposed two ways via which coumaphos can contaminate queen cells. Firstly, the worker bees that secrete the wax used to construct queen cells could have physically contacted coumaphos residues in the brood nest. Secondly, the developing queens may contact coumaphos via feeding by nurse bees (58). The second of the two seems less likely, since trophallaxis plays only a minor role in coumaphos distribution among bees (113). This suggests that in order to mitigate the negative impacts of acaricides on queen bees, beekeepers should frequently change old pesticide-laden combs for new uncontaminated ones.

Some acaricides have also been linked with acute mortality of queen bees. In mite-free colonies, fluvalinate application decreased queen bee survivorship by 22% after 13 months, although this difference was not statistically significant (73). According to Sokol, in colonies treated with 250 mg strips of fluvalinate for six months, the queen ceases to lay eggs and dies. Combs contaminated with fluvalinate caused new queens to lay unfertilised eggs (76 but see 114). It has been reported that 50% of colonies treated with a thymol-oil spray as a means of Varroa control lost their queens (109). Oxalic acid and formic acid have been associated with queen losses (90, 98).

Less research focused on how acaricide application affects the reproductive characteristics of drones. Significantly less drones infested with Varroa mites and treated with Apistan® (fluvalinate) survive the first day of their adult life. Like this, many drones die before reaching sexual maturity. Acaricide treatment also has negative effects on drone weight, mucus glands, number of spermatozoa, sperm viability and seminal vesicles weight (50, 54, 60, 72, but see 107). Exposing drones to recommended label concentrations of coumaphos throughout their development and maturity caused a 50% reduction in spermatozoal production (49).

Even organic treatment methods can leave negative imprints on drone reproductive fitness. Formic acid causes bees to remove their drone brood, delay their drone production and reduces drone survival (88). Likewise, it was hypothesised that thymol treatment may cause a reduction in drone flight activity (107). Acaricide treatment decreases the protein content of drone seminal fluids, probably leading to lower sperm survival (50). In effect, acaricides may cause queens to

mate with an insufficient number of drones of a poor sperm quality. This probably results in an earlier than normal supersedure of the queen bee (115) and adversely impacts colony survivorship and productivity.

Effects on Brood Development

The widespread application of synthetic acaricides, organic acids and homemade acaricidal drugs creates a potentially deadly cocktail of chemicals that can negatively affect bee brood (116). Acaricide residues are ever-present in the hive and the brood can contact them directly in the walls of the wax combs, or can be fed dangerous doses by nurse bees (117). It has been shown that brood raised in wax combs contaminated with coumaphos and fluvalinate have higher mortality, lower longevity, delayed larval development and delayed adult emergence (68, 69). Laboratory studies have shown that acaricides applied at field doses can significantly increase larvae mortality (63). This could severely affect job division in the colony and make the colony less able to respond to abrupt changes in workforce demand such as in times of nectar dearth or when a large portion of the workers is lost for some reason, for example after pesticide poisoning.

Organic acaricides can be equally damaging to the brood. Apilife VAR®, a thymol-based medicament, decreases brood production and causes brood removal (100, 102, 103), especially at high in-hive temperatures (106). Thymol is in particular toxic to larvae (118). This toxicity was attributed to the strong and aromatic vapours the acaricide produces (101). Bee brood in the immediate vicinity of thymol gauze bags is readily removed by the bees (40). Gregorc et al. reported increased cell death in honey bee larvae following the application of oxalic and formic acid (85). By five days, 82% of epithelial cells of the treated larvae were affected, this was then followed by cell death in the entire larval body. Silva-Zacarin et al. further confirmed increased cell death in bee larvae treated with oxalic acid (99). A total of 18.7% of capped brood treated with oxalic acid is removed compared to 13.3% for the controls, and this difference was statistically significant (94). In addition, laboratory studies have shown that a high concentration of formic acid in the hive air (about 2500 ppm) halted oxygen consumption in bee brood. Subsequent *in vivo* studies have shown that treating bee colonies with 20-40ml of 60% oxalic acid reduces the rate of respiration and larval feeding (34). This may explain why colonies treated with oxalic and formic acid tend to have less brood (86, 90, 93, 98) and why bees clear cells close to absorbent pads with formic acid (90).

Effect on Honey Bee Learning

Responses to odour are key for the survival of honey bee colonies, since they play a key role in communication and foraging (111). Fluvalinate and coumaphos were found to impair bee odour learning and discriminatory abilities (77, 119). Whether chronic exposure to fluvalinate or coumaphos under field conditions could affect bee learning and foraging remains unknown.

Effects on Disease Susceptibility

Although acaricidal drugs are meant to alleviate parasite pressure on honey bees, they could also have the opposite effect. Short term, acaricides can efficiently control bee mites and contribute to stronger and healthier bee colonies (112). However, chronically applied sublethal doses that weaken bees could also make colonies more susceptible to other diseases.

Wu et al. (2012) found that bees that developed in combs that contained both coumaphos and fluvalinate residues subsequently suffered from higher infection with *Nosema ceranae.* This may be because bee brood developing in acaricide-laden combs utilize more energy for detoxification purposes which results in lowered immunity (43, 51, 120). The effects may be drastic, since nosematosis often results into colony collapse.

Previous research has shown that high levels of acaricides residue in brood combs prolong the developmental times of brood. Since bees take longer to develop, this provides more time for Varroa mites to reproduce possibly leading to a steeper population growth (69). Although no clear consensus on the effects of *A. mellifera* development time on Varroa population has been reached, it is possible that sublethal acaricide concentrations can paradoxically lead to higher mite population build up over a longer period of time.

Organic acids have been shown to negatively affect the immunity of adult worker bees. For example, formic acid was shown to impact the

proteolytic system in the bee cuticle. Adult bees treated with formic acid had a higher H+ protease and H- protease activity. The cuticle of treated bees showed lower antimicrobial activity. This may have critical implications for bee metabolism and body defence. It is widely believed that as a result of impaired metabolism and body defence, bees treated with formic acid are more susceptible to other serious diseases, namely fungal diseases (89).

Locke et al. showed that shortly after winter treatment with fluvalinate, the tires of the Deformed Wings Virus (DWV) increased for a certain period of time (121). The authors suggested that this may be because the acaricides application weakens the bees and makes them more susceptible to viral infections.

Effects on Colony Strength

The adverse sublethal effects of chemical residues on the queen, drones, adult workers and brood impair colony strength (111). Some acaricides induce aversion in worker bees, retard colony build-up and slow down food intake (91, 102, 122), leading to lower honey yields. For example, the application of thymol and formic acid causes up to 30% decrease of honey production in treated colonies, although the effect was not statistically significant (31, 90). Following the application of thymol or coumaphos, colonies experience a temporary period of stagnation when the amount of brood, adult honey bees and pollen does not increase or increases less than in untreated colonies (100, 101). These temporary stagnations and reductions in honey production may leave a lasting impact and lead to a poorer overwintering and even to colony collapses during the following winter (76, 95, 97). Figure 1 shows how a combination of adverse side effects of acaricides could lead to elevated bee colony losses.

Past research found that the effects of acaricides vary considerably between different colonies. Firstly, the infestation rate of the colony will affects its chances of survival (86). Secondly, Rademacher and Harz pointed out that the tolerability of oxalic acid varies throughout different climates (70). While most researchers in Southern Europe find their colonies to tolerate doses of oxalic acid as high as 7% applied throughout trickling without outstanding negative effects on colony strength, researchers in Northern Europe report that their colonies tolerate much lower doses. This trend could be caused by different susceptibility to chemicals by different bee genotypes. Thirdly, the extent to which acaricide application affects colony strength is probably also dependent on the time of application (40). If the acaricides are used during periods when bees cannot fly outside of their hive, they are less able to rid themselves of residues and thus the effect may be much more serious (97). Lastly, the method of application and dose also has a decisive effect on the subsequent strength of the colony. Bee colonies treated by oxalic acid via trickling or spraying had a lower brood area then those treated by sublimation (28).

Resistance to Acaricides

As acaricides are applied routinely on an annual basis, Varroa mites are gradually building up resistance to some of them (123). Beekeepers are forced to switch to other chemicals (124, 125) which further increases the dependency on chemotherapy. It has also been suggested that by developing resistance to coumaphos, *V. destructor* may simultaneously develop resistance to other chemicals such as amitraz (124). This phenomenon is known as “cross resistance” and could mean that the apicultural industry may run out of chemicals effective against *V. destructor* in the foreseeable future.

V. destructor is persistently exposed to acaricide residues in bee products throughout its life. For example, Varroa mites continue to die 3 months after fluvalinate is applied. This is because fluvalinate is readily absorbed into beeswax and bee bodies and these residues remain toxic to the mites long after the application (80). Other sources claim that bee combs may show acaricidal activities even one year following the application (126, 127) and acaricide contamination can be detected up to seven years after the last application. As a result of this high contamination of beeswax with acaricides, Varroa mites are chronically exposed to the residues for most of their lives and probably develop resistance easier (128). If this really is the case, it is likely that thanks to acaricide residues in bee products, beekeepers are unknowingly selecting for more and more resistant mites.

The toxic effects of acaricides are not limited just to Varroa mites. Chemicals used in the hives target many other inhabitants of the hive, including

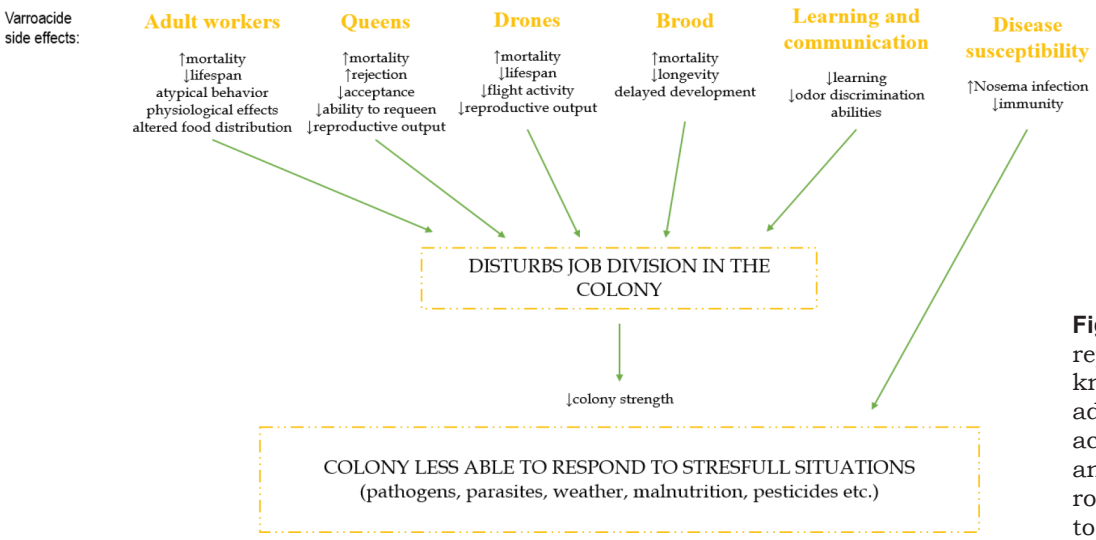

Figure 1: A schematic representation of the known and likely adverse effects of acaricides on bees and their possible roles in contributing to colony losses

commensals and parasites. For example, a wide range of popular acaricides including fluvalinate, thymol and coumaphos exert various effects on the development of the small hive beetle (*Aethina tumida*) (129). This could have yet unknown impacts on the small hive beetle´s populations. For example, the application of these chemicals may accelerate the beetle´s development of cross-resistance to other pesticides. The diverse impacts of acaricides on the development of resistance in other inhabitants of the hive will require large holistic studies in the future.

Deleterious Interactions of Acaricides

Most acaricides persist in bee products for lengthy periods of time (125, 127). A notable exception is amitraz, that is degraded very quickly and so does not accumulate in wax (125, 130). However, this does not necessarily mean that amitraz doesn't leave any residues behind. Within 3 to 4 weeks after application, amitraz breaks down into 2, 4- dimethyl phenyl formamide and 2, 4-dimethylaniline, both of which are environmentally very stable and furthermore the latter is mutagenic, oncogenic and genotoxic (44, 45-48). This shows that even readily degradable chemicals have a potential to cause significant damage to bees.

The accumulation of residues of acaricides such as coumaphos and fluvalinate can exert a lasting negative pressure on bees. Specifically, Johnson et al. demonstrated toxic interactions among the residues of acaricides as well as antimicrobial drugs and fungicides found in bee colonies (131). The toxicity of tau-fluvalinate increased when combined with other medicaments. Bees treated with coumaphos are more susceptible to poisoning by organophosporus pesticides such as parathionethyl, dimethoate, dialifos (55). Likewise, the toxicity of coumaphos increases when applied in conduction with fluvalinate (65) and bees previously treated with the antibiotic oxytetracycline were more susceptible to coumaphos and fluvalinate (132).

Discussion

Studies on the negative effects of acaricides on honey bees are relatively scarce, when compared to the total body of literature on Varroa control and many questions require further research. Beekeepers often apply acaricides at the end of the season after the last honey extraction, to minimise the risk of residues in honey. When bee colonies overwinter, which can last as long as 6 months, they are entirely dependent on the stores they produced during the year. If the medications affected bee stores, this could have a crucial effect on bee colony strength as a whole next spring. A study on humans showed that formic acid, oxalic acid and thymol added to honey significantly affected its taste (133). Could residues over sensory levels harm bees? This, among other questions, remain to be resolved with further research. So far, it has been demonstrated that formic acid induces aversion in worker bees (91, 122) and that formic and oxalic acids affect the pH

of honey stores (81). As of present, little is known about the potential effects of pesticide residues on overwintering bees (134).

Another research topic that will require more attention in the future are the wider implications of chemotherapy for bee breeding. The heavy use of chemotherapy in apiculture may have much more pronounced effects on bee colonies then just what may seem as minute sublethal effects. Elzen et al. demonstrated that the European honey bee (*A. m. ligustica*) was significantly more tolerant to pyrethroids than African honey bees (*A. m. scutellata*) (135). It was suggested that European bees that are managed much more intensively, face selection pressures because of the routine application of synthetic acaricides. It thus seems that the advent chemotherapy in beekeeping significantly altered *A. mellifera* phenotype and maybe even its gene pool. This may subsequently bring serious problems in bee breeding.

Since all of the acaricides in use today have some kind of a negative side effect on bees, it is difficult to recommend the most bee-friendly product that would leave no negative health effects on colonies. The ability of beekeepers to prevent undesirable side effects of acaricides is also restrained by the fact that the manufacturing process of commercially available veterinary drugs for bees is rather non-transparent. Different formulations of the same acaricide can have very different impacts on bees. In order to overcome pest resistance problems, it is common practice now that pesticide manufacturers frequently change the formulation of their products. For example, earlier formulations of fluvalinate used in the 1980s were only slightly toxic to bees, but modern fluvalinate products are considered highly toxic (136). Furthermore, when acaricides are applied to the hive, the amount of active ingredients released may vary enormously between different commercial formulations. Factors such as nest congestion, bee activity and length of exposure may profoundly affect the amount of substance released per unit of time (137). The actual amount of active ingredient distributed through the colony may therefore be different from the amount given on the label. As such, it is hard for beekeepers and bee researchers to predict what side effect may their acaricides have. Thus, with some exaggeration it can be said that every batch of bee medicaments made has its own, unpredictable effects.

In summary, although acaricides can significantly decrease *V. destructor* infestation, they may exert negative effects if the colony is only lightly infested (86). The scale and extend of the negative impacts of acaricides is strongly influenced by factors such as the climatic conditions, the time of the year, the dose and the method of application (26, 27, 28, 70), so reaching consistent results has long been a problem. The negative effects of acaricides can be so cryptic that they can be hard to recognise by beekeepers and only cause acute poisoning if coupled with other stressors (42). Even methods used in organic beekeeping may leave long-term negative impacts on honey bee health. It is therefore suggested that acaricides are only applied as a last resort when the Varroa population reaches damaging levels. A growing number of beekeepers is turning towards zootechnical and biological methods such as drone comb removal, hyperthermia, selecting for Varroa-resistant and Varroa-tolerant bee stock, powder sugar dusting, using combs with smaller cell size and others that do not require the application of chemicals and so do not leave any residue behind and minimalize the risk of the pests developing resistance (5, 130, 138, 139). While often more time consuming and less efficient than conventional drugs, rapid developments are being made that make these methods easier to apply then before (140). Organic beekeepers are organised in a number of societies and are entitled to label their honey as “organic honey”. It has been shown, that the growing interest of beekeepers in organic methods has brought forward a decrease of acaricide residues in bee products (130, 139). Novel organic Varroa control methods will certainly meet more and more popularity in the beekeeping circles in the very near future and contribute to increasing the sustainability of apiculture worldwide.

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VPLIV UMETNIH IN NARAVNIH AKARICIDOV NA ZDRAVJE MEDONOSNIH ČEBEL

E. Tihelka

Povzetek: Čebele so pomemben opraševalec kmetijskih pridelkov in gospodarsko pomemben proizvajalec izdelkov, kot sta med in vosek, ki se uporabljajo v živilski industriji, kozmetiki in medicini. Trenutno je zunanji zajedavec pršica varoja (*Varroa destructor*) eden največjih škodljivcev za čebele v svetu. Brez zdravljenja čebelje družine napadene z varojo večinoma propadejo v nekaj letih. Za ohranjanje nizke populacije pršic varoj čebelarji uporabljajo umetne in naravne akaricide. Najbolj priljubljeni, komercialno dostopni, so amitraz, kumafos, flumetrin, fluvalinat, mravljinčna kislina, oksalna kislina in timol. Ti akaricidi so dokaj poceni in enostavni za uporabo, vendar podaljšana uporaba povzroča hitro razvijanje odpornosti pri pršicah varoja in večjo možnost onesnaženja čebeljih pridelkov. Ostanki akaricidov so po zdravljenju lahko prisotni v visokih koncentracijah v celotnem panju in so jim čebele izpostavljene celo leto. Po uporabi je tudi satje pogosto onesnaženo z visokimi koncentracijami ostankov akaricidov, ki so jim pršice varoja kronično izpostavljene in lahko zaradi tega hitreje razvijejo odpornost. Pregledni članek povzema trenutno znanje o škodljivih učinkih konvencionalnih akaricidov na zdravje čebel. Dokazano je, da imajo številni komercialno dostopni akaricidi in njihove aktivne snovi negativne učinke na razvoj čebel, vplivajo na razmnoževalno sposobnost čebelje matice in zmanjšujejo sposobnost učenja čebel ter dolgoživost in moč kolonije. Akaricidi ne delujejo samo kot posamezne učinkovine, ampak tudi sinergistično, kar lahko dodatno slabo vpliva na zdravje čebelje družine. Nekatera zdravila lahko povzročijo znatno oslabitev čebeljih družin, lahko pa jih tudi naredijo bolj dovzetne za druge bolezni, kot je nozemavost, ali bolj občutljive na slabe vremenske razmere. V kombinaciji z drugimi stresorji lahko akaricidi prispevajo k propadu čebelje družine.

Ključne besede: umetni akaricidi; varoicidi; čebela; *Apis mellifera*; sinergija; ekološko čebelarjenje

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Review Article

SEROTYPE-SPECIFIC AND HAEMADSORPTION PROTEIN OF THE AFRICAN SWINE FEVER VIRUS

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Abstract: This review presents comparative results of simultaneously conducted studies on proteins responsible for the haemadsorption and serotype-specific properties of African swine fever virus (ASFV).

An ASFV gene EP402R (or LMW8-DR) encoding protein CD2v homologous to murine, human or porcine T-cell adhesive receptor was found. The CD2v was shown to be directly involved into a haemadsorption process, and expressed in ASFV-infected cells as a glycoprotein with a molecular weight of approximately 105-110 kDa. In the presence of a glycosylation inhibitor, tunicamycin, its molecular weight is about 42 kDa.

In ASFV-infected cells labeled with ^{3}H-glucosamine or ^{14}C-sodium acetate, a virus-specific major glycoprotein with a molecular weight of 110-140 kDa (gp 110-140) was identified using radioimmunoprecipitation assay. Using ASFV reference strains belonging to seroimmunotypes I-IV and the corresponding antisera active in haemadsorption inhibition assay (HADIA), we determined that gp 110-140 defines the serotype specificity. Genotyping on the basis of the genetic locus encoding the CD2v and a C-type lectin protein also showed a concurrence with the grouping of ASFV isolates and strains based on their seroimmunotypes. Immunization of pigs with the gp 110-140 within liposomes, or a recombinant haemagglutinin (CD2v) protected 67 to 100% of animals from death due to their subsequent infection with homologous virulent ASFV strains.

Based on the physico-chemical and biological characteristics of the gp 110-140 and CD2v it is suggested that they are one and the same virus-specific glycoprotein crucial for induction of the immunological protection against ASF.

Key words: ASFV; seroimmunotypes; serotype; glycoproteins; gp 110-140; CD2v; protectivity

Introduction

African swine fever (ASF) is a contagious, septic viral disease of pigs characterized by fever, toxicosis, hemorrhages, and high mortality rate. The causative agent of the disease is a large DNA virus in the *Asfarviridae* family (1). Domestic and wild pigs (*Sus scrofa*) are all susceptible to ASF virus regardless of the breed or age. The disease can occur in various forms including fulminant, acute, subacute, chronic, and inapparent. The virus can be transmitted from sick animals and virus-carriers by contact, by alimentary and transplacental routs, as well as by soft ticks of the genus *Ornithodoros* (2).

Cells infected with ASFV field isolates are able to adsorb swine erythrocytes (haemadsorption) on their surface (3), a property successfully exploited to differentiate the ASFV from other agents causing diseases with symptoms likely to be confused with those observed in ASF (4), to develop specific techniques for ASFV titration (5), to select attenuated variants *in vitro* (6).

The ability of serum from survivor-carriers to inhibit the haemadsorption reaction in the course of ASFV replication in pig bone marrow (PBM) cultures or swine leukocytes was used in studies

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on the antigenic diversity of the virus. Using haemadsorption inhibition assay (HADIA) and immunological bioassay, Malmquist (7) showed the antigenic diversity of ASFV isolates identified in Africa. Zucas et al. (8) concluded that two or more immunological types circulated in Africa, whereas in European countries and in Cuba, the isolates were antigenically identical. Sanchez-Botija and Ordas confirmed that the European isolates Italy-67, Salamanca and Portugal-60 differed from the African isolates (9). Based on differences determined by HADIA with antisera against some ASFV strains from Katanga, Madeira Island and Portugal (1957 and 1960), Vigario et al. (10) classified the available isolates into three antigenic groups (A, B and C) using Lisbon-57, Lisbon-60, Hinde-2, Rhodesia and Mozambique ASFV as the reference strains. Analysis of more than 100 virulent and attenuated strains provided the basis for ASFV seroimmunological classification (11-13).

ASFV strains are assigned to a respective group based on the data obtained *in vivo* (bioassay based on cross protection of pigs survived after their infection with a virulent strain) or *in vitro* (HADIA). The strains assigned to the same group using results of bioassay and HADIA were combined into nine separate seroimmunotypes with the respective reference strains: I – Lisbon-57 (L-57), II – Congo-49 (C-49), III – Mozambique-78 (M-78), IV – France-32 (F-32), V – TSP-80, VI – TS-7, VII – Uganda, VIII – Rhodesia and Stavropol 01/08 (including other strains of genotype II currently circulating in Eastern Europe), and IX – Davis (14, 15).

Genotyping of ASFV isolates by partial sequencing of the B646L gene that encodes the major capsid protein p72 has identified 23 genotypes (16, 17). HADIA serology provides a measure of ASFV typing that, compared with p72 genotyping, better discriminates biologically pertinent phenotypes. Viruses belonging to one p72 genotype may be serotypically different. For example, ASFV isolates belonging to seroimmunotypes I, II, and IV were all clustered into genotype I. This indicates heterogeneity among ASFV strains isolated on the European continent (18).

This review presents comparative results of simultaneously conducted studies on proteins responsible for the haemadsorption and serotype-specific properties of ASFV.

Haemadsorption protein CD2v

The initial reports on the detection of these proteins were published in the 1990s. Furthermore, an ASFV gene EP402R (also known as LMW8-DR) encoding a polypeptide of 402 amino acids homologous to an adhesive receptor of the T-cells (CD2) of mice, humans and pigs was revealed (19, 20). The expected molecular weight of the non-glycosylated polypeptide was 45.3 kDa. Hydrophilicity profiling shows that this protein is a typical transmembrane protein consisting of four differentiated regions: a hydrophobic leading region at the N terminus of 20 amino acids; a hydrophilic extracellular region of 183 amino acids with 15-16 glycosylation sites; a transmembrane region of 25 amino acids; and a proline-rich cytoplasmic C-terminal region of 174 amino acids. The protein CD2v was shown to be expressed in ASFV infected cells as a glycoprotein of approximately 105 kDa (25). The extracellular region of the protein contains two Ig-like domains, whereas CD2 has three or four such domains (19-24). Deletion of the EP402R had no effect on the rate of virus replication *in vitro*, although the virus lost the ability to induce adsorption of pig erythrocytes on the surface of infected cells, indicating that the ASFV protein CD2v, encoded by EP402R, is associated with the phenomenon of haemadsorption.

Serotype-specific glycoprotein gp 110-140

Detection of the ASFV serotype-specific protein was reported in parallel with publications on the CD2v. Using radioimmunoprecipitation assay, a major virus-specific glycoprotein with a molecular weight of 110-140 kDa was found as dumbbell-shaped band typical for highly-glycosylated proteins (26). For its detection two principal conditions were required: 1) use of the metabolically ^{3}H-glucosamine-labelled proteins derived from lysates of PBM naturally susceptible A-cells infected with ASFV haemadsorbing strains as an antigen source; 2) use of the homologous antisera with high activity in HADIA as an antibody source. In the lysates of PBM A-cells infected with non-haemadsorbing ASFV strains or when using homologous antisera inactive in HADIA (e.g., antisera obtained from pigs inoculated with attenuated ASFV strains, inducing those with the

atypical pattern of haemadsorption), gp 110-140 was not detected (Figure 1) (26).

The above conditions suggest that gp 110-140 is a haemadsorption protein. The serological specificity of gp 110-140 has been determined. While using some homologous components in the radioimmunoprecipitation assay such as the lysate of PBM A-cells infected with seroimmunotype IV ASFV strain F-32 or serotype IV antiserum active in HADIA, the dumbbell-like bands of gp 110-140 manifested as the major ones. In the assays using heterologous components such as lysates of PBM A-cells infected with seroimmunotype IV ASFV strain F-32 or serotype I antiserum active in HADIA, gp 110-140 was not detected in the fluorograms or manifested less intensively as compared to the results of the homologous assay (Figure 2).

A method for quantitative assessment of serological relationship of haemabsorbing ASFV strains based on results obtained as qualitative assessment of gp 110-140 band intensity was developed. Results of radioimmunoprecipitation are to be recorded not through a visual examination of the fluorogram, but by the number of pulses per a minute using a β-counter. ^{3}H-glucosamine-labelled gp 110-140 preparations, derived from haemadsorbing ASFV reference strains of seroimmunotypes I to IV purified with ion-exchange chromatography on DEAE Sephacel, were used as antigens for the quantitative version of the radioimmunoprecipitation procedure. Reference antisera against ASFV serotypes I to IV (activity in HADIA 1: 320-1:640) were used as an antibody source (27-29). After washing the immune complexes precipitated on the

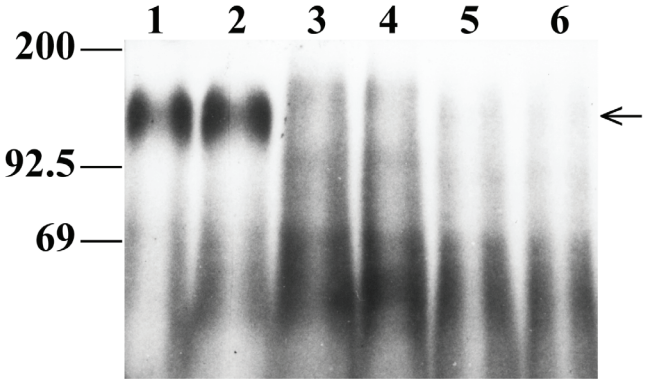

Figure 1: Immunoprecipitation of gp 110-140

A-cells of PBM were infected with ASF virus strains F-32 (tracks 1-4) or FNH (tracks 5 and 6) at the multiplicity of infection (MOI) of 5.0 HAU_{50} or 50% $TCID_{50}$ per cell. One hour post infection, the growth medium was replaced by a glucose-free medium supplemented with 0.2 MBq/ml D-(6-$^{3}H_{1}$) glucosamine hydrochloride. After 16 hours, the cells were harvested at a volume of 1/100 of the original volume, pelleted and extracted in 1% Triton X-100 in 0.02 M Tris-HCl (pH 7.2). The immunoprecipitation was conducted using 1 cm^3 of each cell extract and 0.05 cm^3 of each serotype IV antiserum with activity in HADIA of 1:640 (tracks 1, 2, 5, and 6) or an inactive in HADIA antiserum from pigs inoculated with the attenuated strain FK-135 of seroimmunotype IV (tracks 3 and 4). The immune complexes were sorbed onto 0.1 cm^3 Protein A-Sepharose® CL-4B (GE Healthcare). After washing, the sorbent was boiled in 0.1 cm^3 of electrophoresis buffer. The molecular weight markers (kDa) are shown on the left. The arrow on the right side indicates the gp 110-140 protein (26).

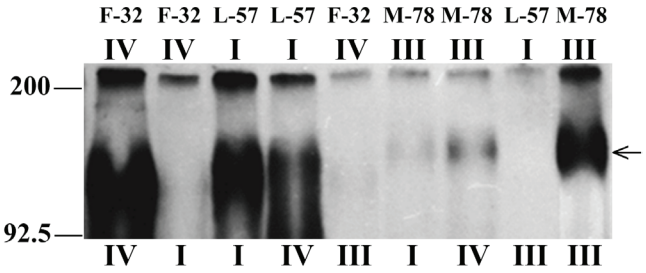

Figure 2: Serological specificity of gp 110-140

A-cells of PBM were infected with ASFV strains L-57, M-78 or F-32 at MOI of 5.0 HAU_{50} per cell. One hour after inoculation, the growth medium was replaced by a glucose-free medium supplemented with 0.2 MBq/cm^3 D-(6-$^{3}H_{1}$) glucosamine hydrochloride. After 16 hours, the cells were harvested at a volume of 1/100 of the original one and extracted in 1% Triton X-100 in 0.02 M Tris-HCl (pH 7.2). The immunoprecipitation was conducted using 1 cm^3 of cells extracts and 0.05 cm^3 of antiserum of serotypes I, III or IV with activity in HADIA of 1:320 to 1:640. The immune complexes were sorbed onto 0.1 cm^3 of Protein A-Sepharose® CL-4B. After washing, the sorbent was boiled in 0.1 cm^3 buffer for electrophoresis. The virus strain and its seroimmunotype are indicated above, and the antiserum serotype is below. The molecular weight markers (kDa) are shown on the left. The arrow on the right indicates the gp 110-140 protein (26).

Protein A-Sepharose® CL-4B (GE Healthcare), equal volumes of the sorbent were boiled in electrophoresis buffer, and the radioactivity of the liquid phase was then determined using a β-counter. Since the incorporation of the ^{3}H-glucosamine into gp 110-140 might depend on the quality of the cell culture, virus titre, and other features, the antigenic relationship was calculated by the relative value, representing percentage of specific binding. The radioactivity of the precipitate resulting from the specific binding of the serotype-specific antiserum and gp 110-140 of the homologous reference ASFV strain was taken as 100 %. The percentage of specific binding obtained with the control sera of intact pigs was not greater than 3 %. The serological relationship of gp 110-140 with serotype-heterologous antisera varied from 20 to 45 % (Table 1) which indicates that the gp 110-140 contains both homologous and heterologous epitopes.

Thus, the serotype specificity of gp 110-140 was confirmed using two versions of radioimmunoprecipitation assay. Moreover, if on fluorograms visual differences in heterologous reactions were significant (Figure 2, tracks IV-IV and IV-I; I-III and III-III), the percentage of specific binding by the results of the scintillation counting was in the range of 20 to 45% (Table 2).

Furthermore, comparative genomics data indicate that the locus containing the adjacent genes of CD2v and C-type lectin protein is one of the most variable loci within ASFV genome (30). They share between 43 and 100% identity with the proteins encoded by the ORFs in other genomes making these amongst the most divergent orthologous clusters (31).

There are some ASF virus strains identical according to HADIA but different by data of immunoassay using animals. Thus, in pigs survived after inoculation with strain F-32 and then infected with isolate Portugal-60 (P-60) acute ASF developed, although, based on the data of HADIA, strain P-60 belongs to serotype IV as well as F-32. Nevertheless, pigs survived after inoculation with strain P-60 survived the subsequent challenge with strain F-32. The assessment of the serological relationship between strain F-32/isolate P-60 gp 110-140 and antisera to seroimmunotypes I-IV as well as antisera to strain P-60 are shown in Table 2 (29). The antiserum to strain P-60 more effectively interacted with gp 110-140 from strains F-32 and P-60, and conversely the degree of antigenic relationship of strain P-60 gp 110-140 with antiserum to F-32 was only 69 % which is consistent by the results of cross-infection of pigs.

Table 1: Antigenic relationship (%) of gp 110-140 preparations of ASFV seroimmunotype I-IV reference strains to serotype I-IV antisera (n=3 to 5) (28)

gp 110-140, (ASFV strain/Seroimmunotype)	Antisera (ASFV strain/Serotype)			
	L-57/I	C-49/II	M-78/III	F-32/IV
L-57/I	100.0±0.0	34.1±9.3	20.2±8.7	35.4±6.0
C-49/II	45.2±6.1	100.0±0.0	32.3±2.4	30.3±4.2
M-78/III	22.3±7.4	31.1±3.2	100.0±0.0	27.3±2.4
F-32/IV	26.1±2.3	27.3±7.2	27.3±5.0	100.0±0.0

Table 2: Antigenic relationship (%) of gp 110-140 from strain F-32 or isolate P-60 to serotype I-IV antisera and isolate P-60 (29)

gp 110-140, (ASFV strain or isolate/ Seroimmunotype)	Antisera (ASFV strain or isolate/Serotype)				
	L-57/I	C-49/II	M-78/III	F-32/IV	P-60/IV
F-32/IV	31.3	27.8	27.4	100.0	120.0
P-60/*	28.3	29.4	42.6	69.0	100.0

* - the isolate is currently included into independent group (by seroimmunological classification), which includes isolates whose serotype obtained by HADIA did not correlate with the results of immunobiological test.

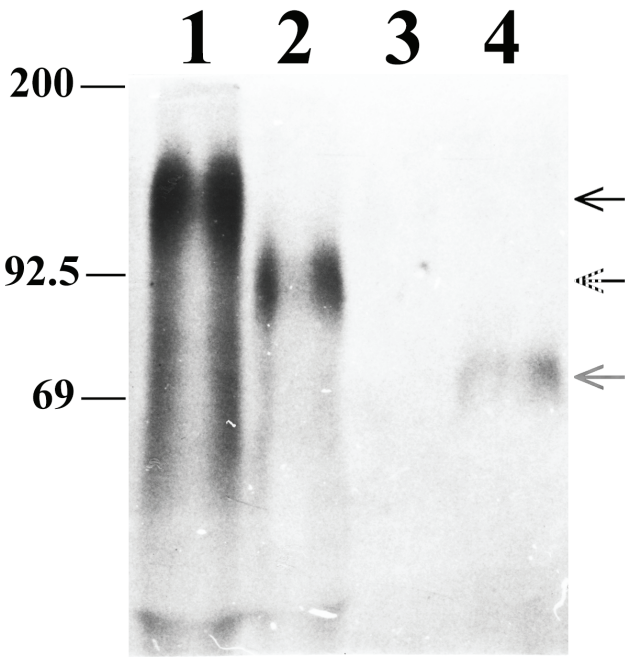

Figure 3: Effect of glycosylation inhibitors on the molecular weight of gp 110-140 (fluorogram)

A-cells of PBM were infected with haemadsorbing ASFV strain F-32 at MOI 5.0 HAU_{50} per cell. One hour post infection, the growth medium was replaced by a glucose-free medium supplemented with 0.2 MBq/cm^3 D-(6-$^3H_1$) glucosamine hydrochloride and the glycosylation inhibitor (tracks 2, 3 and 4). After 16 hours, the cells were harvested at a volume of 1/100 of the original one and extracted in 1% Triton X-100 in 0.02 M Tris-HCl (pH 7.2). The immunoprecipitation assay was carried out using 1 cm^3 of each cell extract and 0.05 cm^3 of serotype IV antiserum with activity in HADIA of 1:640. The immune complexes were sorbed onto 0.1 cm^3 Protein A-Sepharose® CL-4B. After washing, the sorbent was boiled in 0.1 cm^3 of electrophoresis buffer. The fluorogram: 1 - control, without inhibitor, 2 – with swainsonine (1 μg/cm^3), 3 - with tunicamycin (1 μg/ cm^3), 4 - with monensin (0.5 μg/cm^3). The molecular weight markers (kDa) are shown on the left. The arrows on the right side indicate the gp 110-140 protein (26).

Physicochemical characteristics of gp 110-140 and CD2v

Also, some physical and chemical properties of gp 110-140 were tested. Thus, some differences in gp 110-140 average molecular weight among seroimmunotype I-IV ASFV reference strains were determined: for L-57 it was 126 kDa, for C-73 – 130 kDa, for M-78 – 135 kDa, and for F-32 – 115-120 kDa (13, 27). The above difference was assumed to be associated with the quantity and the size of the carbohydrate chains on the glycoprotein molecule. As determined using glycosylation inhibitors and glycoprotein trimming (tunicamycin, swainsonine, and monensin), oligosaccharides represent at least 50 % of the gp 110-140 weight (Figure 3).

In the presence of swainsonine, the molecular weight of the serotype-specific glycoprotein decreased to 95 kDa (Figure 3, track 2), and of monensin to 70 kDa (Figure 3, track 4). Tunicamycin completely blocked N-glycosylation, and radiolabeled ^3H-glucosamine was not incorporated into this molecule (Figure 3, track 3) (27). It is noteworthy that CD2v is expressed as a glycoprotein of approximately 105-110 kDa in ASFV infected cells, and in the presence of tynicamycin acting as a glycosylation inhibitor, the molecular weight of CD2v is about 42 kDa (23, 25). The presence of a formidable «carbohydrate cloud» on gp 110-140 could be a significant obstacle for the immunorecognition of ASFV infected target cells by T-helper cells, cytotoxic T-lymphocytes and antibodies. Thus, the increased percentage of virus-specific cytotoxic T-lymphocyte-mediated lysis of PBM A-cells infected with haemadsorbing ASFV strains in the presence of the glycosylation inhibitor tunicamycin was evident (32). The gp 110-140 proved to be sensitive to protease E treatment and resistant to the treatment with trypsin, endoglycosidase D, endoglycosidase H and/or a number of exoglycosidases (28). Furthermore, similar results were obtained when studying CD2v (25). The CD2vHA plasmid was transfected into Vero cells infected with the BA71V ASFV isolate. At 16 h post-transfection, CD2vHA was immunoprecipitated from cell extracts with anti-HA antisera, and the immunoprecipitates were digested with endo-H or endo-F. A proportion of the high molecular mass form of CD2vHA was resistant to digestion with endo-H, but digestion with endo-F resulted in the disappearance of this form and appearance of a smaller band.

At a single-cycle infection, when the MOI of PBM A-cells infected with strain F-32 was 3 to 10 HAU_{50} per cell, the gp 110-140 was detected starting from 6 to 8 hours post infection (p.i.) with maximum on the hour 10 to 12 p.i., whereas the budding of virions was seen 12 to 14 hours p.i. at the earliest (28). To follow CD2v localization during ASFV infection, CD2vHA was expressed

in ASFV-infected Vero cells. The cells were fixed at 4, 12, 16 and 20 hours p.i. and CD2vHA was detected with indirect immunofluorescence using a rat monoclonal antibody against the HA epitope and a fluorescent-labelled goat anti-rat secondary antibody. CD2v is expressed late in infection and therefore no expression could be detected at hour 4. From 12 hours p.i., a punctate pattern was detectable around the virus factories (25). The molecular weights as well as the similarity of the results with respect to the effect of the glycosylation inhibitors and endoglycosidases on gp 110-140 and CD2v, and also the dynamics of their detection in the infected cells, indicate that it is one and the same protein.

An isoelectric point in gp 110-140 was determined. To achieve the simultaneous radiolabelling of the proteins for amino acids and carbohydrate components, ^{14}C-sodium acetate was used. The isoelectric focusing in granulated gel showed that gp 110-140 had pI=4.3 to 4.8, which is the lowest pI level among all of the virus-specific proteins (Figure 4) (27).

Due to this property, a method for gp 110-140 purification was developed based on the glycoprotein separation from other virus-specific proteins through preparative isoelectric focusing in a granulated gel Ultradex, and from cell proteins through subsequent affine chromatography on an immunosorbent prepared using immunoglobulins taken from homologous antisera.

Protective properties of gp 110-140 and CD2v

Inoculation of attenuated ASFV strains to pigs is known to protect animals from the disease and/or their death after their infection with some homologous virulent isolates or strains belonging to the same seroimmunological group (33-37).

Among all the known ASFV proteins, only gp 110-140 has a property of serotype specificity. Therefore, some experiments on pig immunization using preparations of purified gp 110-140 were performed. To enhance the immunogenicity, the gp 110-140 was incorporated into liposomes. As a result, the fourfold immunization with liposome-incorporated gp 110-140 was shown to protect two of the three challenged animals from death, but not from the disease (38). The pigs immunized with endoglycosidase F deglycosilated gp 110-140 died due to challenge (39). These findings suggest gp 110-140 to be considered as one of the key components required for designing a new generation of protective preparations against ASFV using more effective approaches allowing protective epitopes to affect the porcine immune system.

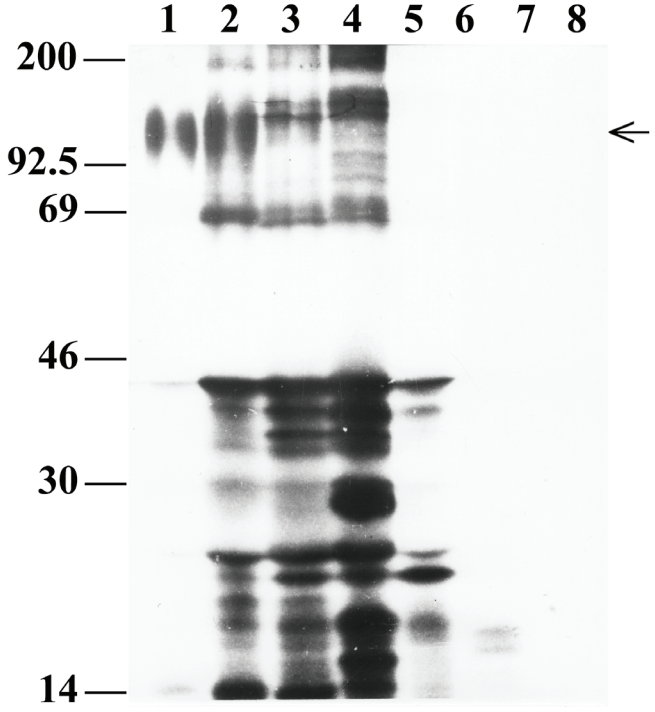

Figure 4: Electrophoregram of ASFV proteins separated with isoelectric focusing

PBM A-cells were infected with haemadsorbing ASFV strain F-32 at MOI of 5.0 HAU_{50} per cell. One hour p.i., the medium was replaced and 0.1 MBq/cm^3 14^C-sodium acetate was added. After 16 hours, the cells were harvested at a volume of 1/100 of the original one and extracted in 1% Triton X-100 in 0.02 M Tris-HCl (pH 7.2). The isoelectric focusing of the extracted protein was carried out in granulated gel Ultradex with 1% solution of Servalites 4 to 9. The solutions of each fraction were dialysed against 1 mM Tris-HCl (pH 7.2), and incubated with 0.05 cm^3 of serotype IV antiserum (HADIA activity of 1:80 to 1:320). The immune complexes were adsorbed onto 0.1 cm^3 Protein A-Sepharose® CL-4B. After washing, the sorbent was boiled in 0.1 cm^3 buffer for electrophoresis. Tracks 1 to 8 show fractions with pH 3.53, 4.27, 4.72, 5.19, 5.83, 6.45, 7.25, and 8.26, respectively. The molecular weight markers (kDa) are shown on the left. The arrow on the right side indicates the gp 110-140 (27).

The similar results were obtained with CD2v. A recombinant baculovirus containing CD2v was constructed. The pigs immunized with the recombinant protein induced antibodies inhibiting haemadsorption and thus were protected from infection with a homologous virulent virus strain (24, 40). In our opinion, a relatively low molecular weight of the recombinant baculovirus CD2v (75 kDa) was due to incomplete glycosylation in the insect cells. Immunization of pigs with the recombinant DNA construction containing the genes of p30, p54 and the extracellular domain of CD2v protected 67 % of pigs infected with a virulent homologous ASFV strain against the disease (41, 42).

Conclusions

Antigenic heterogeneity of ASFV encouraged researchers to develop methods for its differentiation.

Using a collection of monoclonal antibodies that provided detection of 10 ASFV proteins, Garcia-Barreno et al. (43) could reveal some binding properties of proteins of 23 ASFV isolates from Africa, Europe and America, and classify them into six groups. Unfortunately, these findings have not been followed on.

Molecular and genetic methods opened up new opportunities. Mapping of the genome of ASFV isolates using restriction analysis confirmed their high level of heterogeneity. Using eight various restriction enzymes, Wesley and Tuthill (44) divided nine field ASFV isolates into four major groups: (i) the East African isolates Hinde1 and Uganda4, (ii) the Tengani isolate from Malawi, (iii) the Spencer isolate from South Africa, and (iv) the Lisbon/60, Madrid/75, Dominican Republic/78, Haiti/78 and Cameroon isolates. The genomes of the isolates from Cameroon, West & Southwest Africa and Europe have been demonstrated to be similar to each other in many respects, whereas the genomes of the isolates from East and Southeast Africa are distinguished from those of the above isolates and also different from each other (45-48).

A method for ASFV isolate genotyping was developed in 2003 based on the partial sequencing of a 478-nucleotide fragment of the C-terminal region of the p72-encoding gene, which allowed classifying the examined isolates into 23 genogroups (16, 49, 50). The further analysis of other variable regions of the genome of ASFV isolates provided dividing of the isolates which previously were classified into a single group based on the data obtained from p72-encoding gene sequencing, into 19 subgroups (51). The subsequent investigations led to the conclusion that care should be taken when constructing phylogenetic tree between ASFV isolates using a small number of genes (52).

Nevertheless, a relationship between the p72-based genotyping and the seroimmunotype classification was investigated. Great genotype and seroimmunotype diversity of ASFV was found in a relatively limited area in the South-East Africa. Significant was the fact that some ASFV genotypic clusters incorporate isolates belonging to different seroimmunotypes. Thus, ASFV isolates belonging to seroimmunotypes I, II and IV were all clustered only within genotype I. However, the ASFV isolate Spenser, that belongs to seroimmunotype II, was genotypically different from the genotype I as well as from all other 22 genotypes (18). Though the genotyping based on the sequence of the p72-encoding gene is a useful tool for phylogenetic analysis of the ASFV isolates, it is irrelevant to the biological features of the virus. Therefore, seroimmunotype classification based on the serological and protective characteristics is important for proper virus characterization and classification.

Following on from that consideration, it was established that genotyping on the basis of the genetic locus encoding the CD2v and a C-type lectin protein is in agreement with the grouping of ASFV isolates and strains based on their seroimmunotypes (53). In other words, the researchers approached the unification of known ASFV classifications by genotype (using CD2v and C-type lectin protein) and ASFV immunological properties.

Thus, on the one hand, using the immunochemical methods, the gp 110-140 was identified as the haemadsorption-related serotype-specific protein, and, on the other hand, using the molecular biology methods, the CD2v was identified as the protein associated with the haemadsorbing characteristics. Taken together, this paper presents data demonstrating that the gp 110-140 and the CD2v are obviously one and the same glycoprotein, crucial for induction of the immunological protection against ASF. Taking into

account the continuing studies on the ASFV CD2v protein properties and functional significance, the presented data on the serotype-specific gp 110-140 contribute to an assessment of its significance in the creation of a unified classification of ASFV isolates, understanding of its role in the immune response, and in the development of the candidate vaccines.

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SEROTIPNO-SPECIFIČEN IN HEMADSORPCIJSKI PROTEIN VIRUSA AFRIŠKE PRAŠIČJE KUGE

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Povzetek: Pregledni članek predstavlja primerjavo rezultatov sočasno izvedenih raziskav o beljakovinah, ki so pomembne za hemadsorpcijo in serotipno specifične lastnosti virusa afriške prašičje kuge (ASFV; iz angl. african swine fever virus). Pri virusu ASFV je bil odkrit gen EP402R (imenovan tudi LMW8-DR), ki kodira beljakovino CD2v, ki je homologna glodavskemu, človeškemu in prašičjemu T-celičnemu adhezivnemu receptorju. Pokazalo se je, da je CD2v neposredno vpletena v proces hemadsorpcije in je izražena v celicah, okuženih z ASFV kot glikoprotein z molekulsko maso okrog 105-110 kDa. V prisotnosti zaviralca glikozilacije tunicamicina je njegova molekulska masa približno 42 kDa. V celicah, okuženih z ASFV, označenih s ^{3}H-glukozaminom ali ^{14}C-natrijevim acetatom, je bil s testom radioimunoprecipitacije ugotovljen virusno specifični osrednji glikoprotein z molekulsko maso 110-140 kDa (gp 110-140). Z uporabo referenčnih sevov ASFV, ki pripadajo seroimunotipom I-IV, in ustreznim antiserumom, ki so bili aktivni pri preizkusu zaviranja hemadsorpcije (HADIA), smo ugotovili, da gp 110-140 določa specifičnost serotipa. Genotipizacija na osnovi genskega lokusa, ki kodira CD2v in C-tip lektinske beljakovine, je pokazala soizražanje s skupino izolatov in sevov ASFV na podlagi njihovih seroimunotipov. Imunizacija prašičev z gp 110-140 v liposomih ali z rekombinantnim hemaglutininom (CD2v) je zaščitila od 67 do 100 odstotkov živali pred smrtjo zaradi njihove naknadne okužbe z virulentnimi sevi ASFV. Na podlagi fizikalno-kemičnih in bioloških značilnosti beljakovin gp 110-140 in CD2v menimo, da gre za isti virusni glikoprotein, ki je ključnega pomena za vzpodbuditev imunološke zaščite pred ASF.

Ključne besede: ASFV; seroimunotipi; serotip; glikoproteini; gp 110-140; CD2v; zaščita

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Original Research Article

EXPRESSION AND BIOLOGICAL ACTIVITY OF GHRELIN, OBESTATIN, AND LEPTIN IN DEFERENT DUCTS OF THE BULL AND RAM

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Abstract: It is known that ghrelin, obestatin and leptin are involved in many biological processes such as appetite-energy regulation, growth hormone release and cell proliferation. In addition to these biological activities of in human and rodents, direct effects of these hormones on reproductive functions and regulation of smooth muscle contractions have gained importance in recent years. In mammals, gonadal functions are regulated by using a complex network of autocrine, paracrine and endocrine signals. The signals involved in the control of energy balance regulate reproductive functions by acting on different hypothalamic pituitary-gonadal axis. The underlying molecular mechanism of gonad is poorly understood and appears to be controlled on genetic, environmental, and hormonal factors. Ghrelin, obestatin and leptin, three recently discovered hormones, are especially coexpressed in endocrine cells. The purpose of this investigation was to examine the immunohistochemical expression and potential biological activity of bull and ram deferent ducts (DD) in relation to the tissue concentration of ghrelin, obestatin and leptin. Ghrelin, obestatin and leptin expression observed in the DD sections were restricted to the cytoplasm of the epithelial and muscle cells. Both ghrelin and leptin expressions were found in smooth muscle cells in muscular layer of DD and smooth muscle cells of blood vessels, and obestatin expression was found in the basal epithelial cells of the luminal epithelium. In conclusion, ghrelin, obestatin and leptin were expressed in varying concentrations in epithelial and muscle cells as well as blood vessels of the deferent ducts of both species. Given the multifunctional biological roles of these peptide hormones, we can suggest that these hormones function in the contraction and hypertrophy of smooth muscles, proliferation or apoptosis of epithelial and muscle cells in DD.

Key words: deferent ducts; ghrelin; leptin; obestatin; ruminant; physiological function

Introduction

In mammals, gonadal functions are regulated by using a complex network of autocrine, paracrine and endocrine signals (1). The central and peripheral endocrine signals involved in the control of energy balance regulate reproductive functions by acting on different hypothalamic pituitary-gonadal axons, thus they linkup between energy homeostasis and fertility (2).

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Ghrelin, an orphigenic peptide that regulates energy homeostasis, is an endogenous 28 amino acid ligand of the growth hormone secretagogue receptor (GHS-R) (3). Ghrelin was first reported to be excreted from the stomach and it is localized in many peripheral organs including the brain, kidney, pancreas, placenta, ovary, uterus and testis together with GHS-R (4, 5, 6). In addition, ghrelin is a hormone that stimulates food intake by acting with the hypothalamus. This hormone also fulfills multiple endocrine and non-endocrine functions such as regulation of cardiovascular functions and metabolic events such as gastric

motility and acid secretion, glucose regulation and insulin secretion, and cell proliferation (1, 7). In recent years, some researches have also reported that ghrelin is involved in the regulation of lactation, pregnancy and reproductive functions, and gonadal activities are critically affected by local expression of ghrelin and its receptor (8, 9). In particular, ghrelin may affect the male reproductive axis in situations such as energy deficiency (10). Another hormone that participates in the gonadal axis and metabolic regulation is obestatin (11). Obestatin is a 23 amino acid peptide hormone produced by the gastrointestinal tract. It is produced by the enzymatic division of pre-pro-ghrelin (12). Besides, this hormone is also synthesized from the liver, pancreas, adipose tissue, spleen and mammary gland (6, 9). The function of obestatin in the body is still controversial.

In recent studies, obestatin and ghrelin have been reported to play a functional role in the regulation of metabolic and reproductive functions together. Obestatin and ghrelin, which play a key role in growth axis and body weight homeostasis, partly regulate reproductive function by paracrine or autocrine pathways. In general, obestatin and ghrelin are functional antagonists of each other (9). Afsar et al. (14) have shown that obestatin activates releasing of growth hormone (GH) in contrast to ghrelin in their in vitro studies.

Leptin, a 167 amino acid hormonal protein of the obesity gene, is a polypeptide hormone in the single chain form. It is produced mainly in adipocyte and regulates normal sexual maturation and reproductive functions by autocrine and paracrine effect. It is also synthesized from the liver, stomach, mammary gland, bone marrow, bowel, ovary, testis, skeletal muscle, placenta and fundus of stomach. It is also involved in regulation of food intake and energy balance. Moreover, leptin promotes development of reproductive organs and positively affects the reproductive activity in males (13, 14).

Following spermatogenesis in mammals, spermatozoa pass through the efferent channels and leave from the testis, and come into epididymis. Spermatozoa undergo post-testicular maturation phases such as mobility and capacitation in the epididymis. During the ejaculation phase, a spermatozoon passes through the urethra by rhythmic contractions of the deferent duct (DD) (15).

The histomorphological structure of DD has already been investigated in detail in various mammalian species (15, 16, 17). On the other hand, studies on the expression of gherelin, obestatin and leptin in the male genital tract are quite limited in all mammalian species including human, and these studies have mostly focused on testis and epididymis. Studies of the expression and localization of these factors in DD are limited mostly to humans and rats (16), and only little information is available for other species. In this context, we have detected no study of the cellular localization and expression of ghrelin, obestatin and leptin in bull and ram. Therefore, the aims of the present study were to identify the localization and expression of these peptide hormones in the bull and ram DD by using immunohistochemistry to help us better understand their physiological roles in these two species, and to compare possible differences regarding other species (18,19,21,22).

Materials and methods

Tissue samples

In the study, samples of eight healthy and adult bulls and eight rams, which were slaughtered in local slaughterhouses in Diyarbakır province, were used. Samples were fixed in the Bouin's fixative for 12 hours, passed through graded series of ethanol, methyl benzoate and benzene, and finally embedded in paraffin wax. Serial sections containing at least three tissue samples with a thickness of 5 µm were cut from each paraffin block (17). Three slides were prepared to demonstrate ghrelin, obestatin and leptin expressions immunohistochemically.

Immunohistochemistry

The Avidin-Biotin-Peroxidase Complex (ABC-code: 85-9043, Histostain Plus Bulk Kit, Zymed, South San Francisco, CA, USA) procedure was used for the immunohistochemical staining method. Following the deparaffinization and rehydration of the slides, the sections were washed in distilled water. Sections were incubated with 3% hydrogen peroxide prepared with methanol for 30 min for the inactivation of endogenous peroxidase, and washed with 0.01 M phosphate buffered saline (PBS; pH 7.4) for 3x5 min. Sections were boiled

in citrate buffer (pH 6.0) solution for 20 min for antigen retrieval in the microwave. They were cooled and washed in (PBS) for 3x5 min. Prior to the addition of the primary antibodies, the slides were incubated with a blocking UltraVision blot (Ultra V Block, Histostain Plus Bulk Kit; Zymed) for 15 min to block non-specific binding. The samples were then incubated with 1/100 diluted anti-ghrelin primary antibody (Rabbit polyclonal to Ghrelin-Anti-Ghrelin antibody, Abcam, cat no: ab129383), 1/200 diluted anti-obestatin primary antibody (Rabbit polyclonal to Obestatin- Antibody, cat no: ab41704) and 1/100 diluted anti-leptin primary antibody (Rabbit polyclonal to Leptin Anti-Leptin antibody, cat no: ab117751) overnight at + 4°C. At the end of the incubation period, the sections were washed in 0.01M PBS for 3x5 min. Then, sections were incubated with biotinylated secondary antibody for 20 min at room temperature and washed in 0.01M PBS for 3x5 min. Sections were incubated with streptavidin-peroxidase (HRP) for 20 min and washed in 0.01M PBS for 3x5 min, followed by incubation in the diaminobenzidine (DAP) chromogen for 5-10 min to demonstrate antigen-antibody reactions, washed with distilled water and counterstained with Gill's Hematoxylin for 2 minutes. After passing through the ethanol series and dehydrated, the sections were cleared with xylene and covered with mounting medium (1079610-500-Merck) (18) and cover slips. The specificity of immunohistochemical procedures was assessed using negative and positive control. Stomach (abomasum) sections of bull and ram were used as positive controls. For the negative controls, the slides were incubated with PBS instead of the primary antibodies. All samples were treated with an identical protocol. The slides were photographed by using research microscope Nikon Eclipse E400 (Nikon, Tokyo, Japan) equipped with a digital camera (Nikon Coolpix 4500).

Semiquantitative evaluations

Immunoreactivities of ghrelin, obestatin and leptin were determined by semiquantitative scoring method (IS) based on the staining intensity (18, 19). IS reflected the positive staining intensity in the membranes, cytoplasm and nuclei of the cells. Staining intensity scores were evaluated as follows: - negative; + weak; ++ moderate; +++ strong. The IS of the immunostaining reactions in the cells was evaluated by two independent investigators (B.G.S., H.S.) and the average score of the two observers was estimated. The expressions of ghrelin, obestatin and leptin in the DD were observed at x20 and x40 magnifications. Three randomly selected areas for each section were evaluated in each region of the DD (epithelium, connective tissue, muscle and adventitia). Furthermore, following the independent evaluation of the immunohistochemical staining, kappa (κ) statistics was used to determine inter-observer agreement. Inter-observer variability was estimated by comparing the visual scores of two researchers. The results were presented separately for epithelial cells, stromal cells, muscle cells, blood vessels and nerves.

Results

Ghrelin, obestatin and leptin immunoreactivities, which were observed in the DD sections of bulls and rams were restricted to the cytoplasm of epithelial and muscle cells. Immunoreaction was observed for ghrelin, obestatin and leptin in the positive controls, while no staining was determined in the negative controls (Figs. 1a,b,c).

Ghrelin. In bulls and rams, ghrelin was strongly expressed in the longitudinal and circular muscular layers of DD and media layer of blood vessels. On the contrary, ghrelin expression was not observed in the luminal epithelium, stromal cells, and nerve plexus in both species (Figs. 2a,b,c and 3a, b) (Table 1).

Obestatin. Expression was strong in the basal cells of the luminal epithelium in both bulls and rams, but weak in the ciliated epithelial cells. In addition, there was a weak expression of obestatin in the connective tissue cells surrounding the adventitial layer of DD in both species. Obestatin immunoreactivity was not determined in the smooth muscle cells of muscular layer, stromal cells of DD and nerve plexuses (Figs. 4a,b and 5a,b,c) (Table 1).

Leptin. In bulls and rams, leptin expression was fairly strong in smooth muscle cells of media layers of blood vessels and muscular layer of DD, and it was moderate in some stromal cells. On the other hand, leptin expression was not detected in luminal epithelial cells, the cells surrounding the adventitia, and nerve plexuses (Figs. 6a,b and 7a,b) (Table 1).

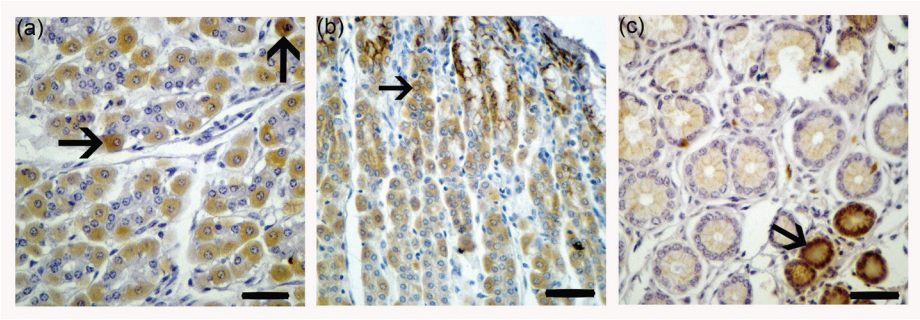

Figure 1: Positive control of the abomasum. (a) expression of ghrelin in the parietal cells (arrows) of bull fundus; (b) expression of obestatin in the parietal cells (arrows) of ram fundus; (c) expression of leptin in the gland epithelial cells (arrows) of bull cardia. Bar: 25 µm (original magnification, x40)

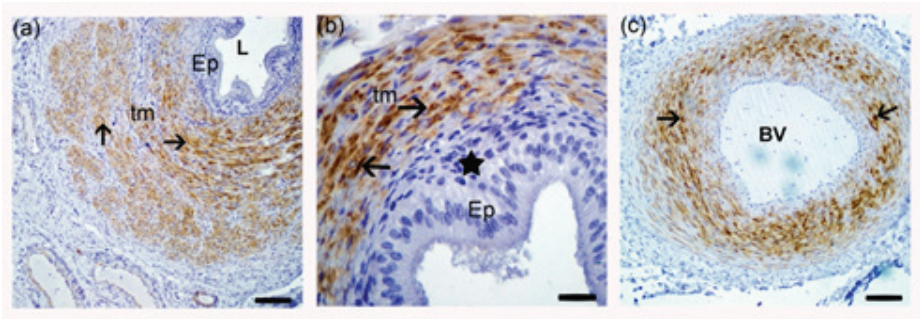

Figure 2: Immunohistochemical expression of ghrelin in the bull DD (a-c; Bar: 50 µm, b; Bar: 25 µm, original magnification, x20 and x40, respectively). (a,b) Strong ghrelin immunoreactivity was observed in smooth muscle cells (arrow) of muscular layer; (b) No immunostaining was detected in stromal cells (asterisk); (c) Strong ghrelin immunostaining was detected in smooth muscle cells (arrow) of blood vessels. L: lumen, Ep: luminal epithelium, tm: tunica muscularis, BV: blood vessel

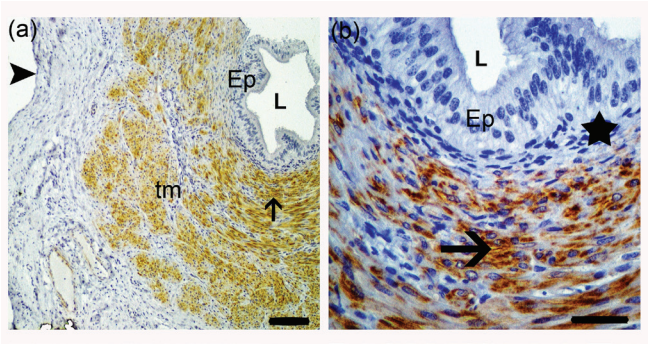

Figure 3: Immunohistochemical expression of ghrelin in the ram DD (a; Bar: 50 µm, b; Bar: 25 µm, original magnification, x20 and x40, respectively). (a,b) Ghrelin immunoreactivity was strong in smooth muscle cells of muscular layer (arrow). (b) No immunostaining was detected in stromal cells (asterisk) and advetitial cells (arrowhead). L: lumen, Ep: luminal epithelium, tm: tunica muscularis, asterisk: stroma

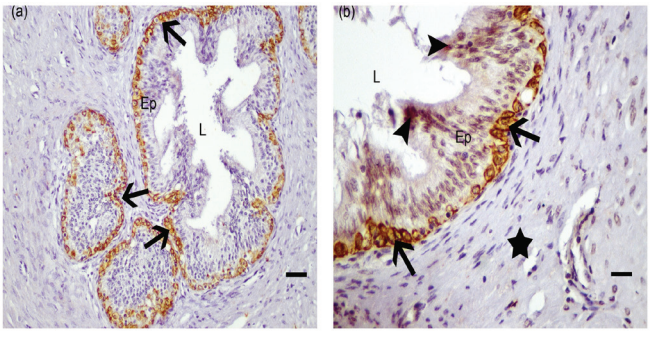

Figure 4: Immunohistochemical expression of obestatin in the bull DD (a; Bar: 50 µm, b; Bar: 25 µm, original magnification, x20 and x40, respectively). (a,b) While weak obestatin immunoreactivity was observed in ciliated epithelial cells (arrowhead), strong obestatin immunostaining was detected in basal cells (arrow) of the epithelium. L: lumen, Ep: luminal epithelium, tm: tunica muscularis, asterisk: stroma

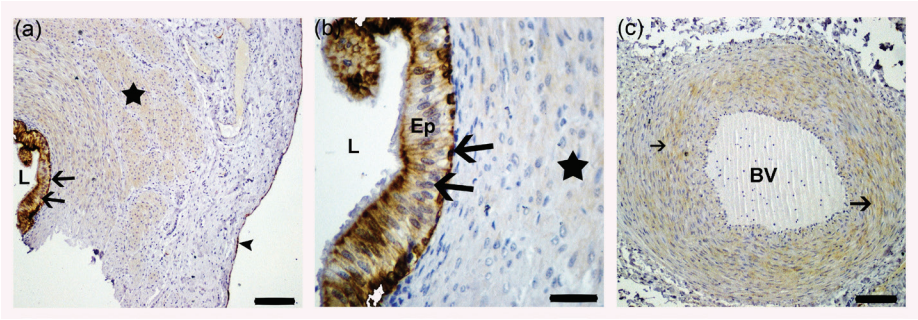

Figure 5: Immunohistochemical expression of obestatin in the ram DD (a-c; Bar: 50 µm, b; Bar: 25 µm, original magnification, x20 and x40, respectively). (a,b) Obestatin immunoreactivity was strongly expressed in basal cells (arrow) of luminal epithelium; (c) Weak obestatin immunostaining was observed in smooth muscle cells (arrow) of blood vessels. L: lumen, Ep: luminal epithelium, tm: tunica muscularis, BV: blood vessel, asterisk: muscle layer

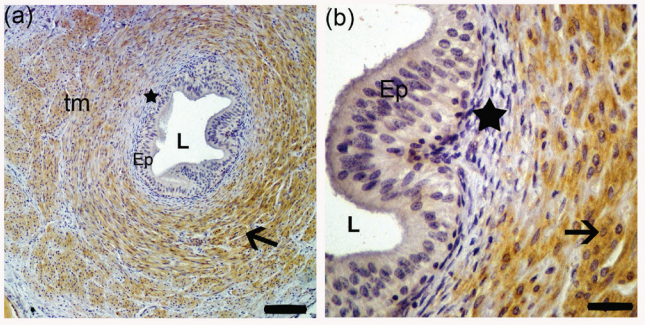

Figure 6: Immunohistochemical expression of leptin in the bull DD (a; Bar: 50 µm, b; Bar: 25 µm, original magnification, x20 and x40, respectively). (a,b) Strong leptin immunoreactivity was observed in smooth muscle cells (arrow) of muscular layer. L: lumen, Ep: luminal epithelium, tm: tunica muscularis, asterisk: stroma

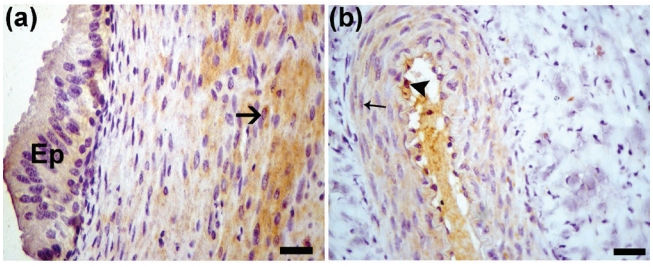

Figure 7: Immunohistochemical expression of leptin in the ram DD (a; Bar: 25 µm, b; Bar: 50 µm, original magnification, x40 and x20, respectively). (a) Strong leptin immunostaining was detected in smooth muscle cells of muscular layer (thick arrow); (b) Strong leptin immunoreactivity was determined in endothelial (arrowhead) and smooth muscle cells (thin arrow) of the blood vessels. L: lumen, Ep: luminal epithelium, tm: tunica muscularis, BV: blood vessel, asterisk: stroma

Table 1: Scoring for immunostaining of ghrelin, obestatin and leptin in the bull and ram deferent ducts

Antibodies	Animals	Ductus Deferens				Blood vessels layers		Nerve plexuses
		Tunica mucosa		Tunica muscularis	Adventitia	İntima	Media	
		Epithelium	Stroma					
Ghrelin	Bull	-	-	+++	-	-	+++	-
	Ram	-	-	+++	-	-	+++	-
Obestatin	Bull	+++	-	-	+	-	-	-
	Ram	+++	-	-	+	-	-	-
Leptin	Bull	-	++	+++	-	+++	+++	-
	Ram	-	++	+++	-	+++	+++	-

Staining intensity: – negative, + weak, ++ moderate, +++ strong

Discussion

In the present study, we showed that both ghrelin and leptin expressions in bulls and rams are strong in muscular layer of DD and smooth muscle cells of blood vessels. On the contrary, obestatin expression is strong in the basal epithelial cells of the epithelial layer. Fertility is controlled by a multi-hormonal effect in all mammals. Any functional defect in the components of this hormonal complex directly affects the reproduction. Recent researches have revealed that ghrelin, obestatin and leptin produced from the adipose tissue and gastrointestinal tract are new members of this hormonal complex. These hormones acting on hypothalamic-pituitary-gonadal axis cause various effects on reproductive functions as well as carbohydrate and fat metabolism and have effects on appetite (20).

The epididymis and DD, part of the epididymis, are a crucial region for the maturation of sperm. It is plausible that these peptides that meet the sperm before their access to the epididymis may influence post-gonadal sperm modifications, such as motility, egg binding and penetration (18). In support, clues on the influence of ghrelin, obestatin and leptin on rat and human sperm motility are available in the literature (18, 19, 21, 22). It was reported that ghrelin and obestatin originate from the same gene, even though in different tissues (18). This discovery could support, at least in part, our observation that ghrelin and obestatin are not necessarily expressed together, as observed in epididymis.

In some studies, correlation and localization results among ghrelin, obestatin and leptin expressions in testicular cells, and the effects of each peptide on reproduction have led to the production of diverse ideas (14). There are studies on the localization and functions of ghrelin, obestatin and leptin in female and male genital systems of mammals including humans (14, 18, 19). However, these studies have mostly focused on the female genital tract (1, 2). Studies on ghrelin, obestatin and leptin in the male genital system have been focused on testis and epididymis in humans and rats (14, 18, 21, 22). A limited number of studies on the localization and biological functions of these peptide hormones have been found in the testes and epididymis in domestic mammals (19).

Ghrelin is expressed by Leydig and Sertoli cells of testis and this hormone can regulate spermatogenesis by autocrine and/or paracrine pathways (21). In rats, ghrelin expression was positive in the apical parts of epithelial cells of the seminal vesicle, and was negative in the connective tissue (23). In addition, it has been reported that ghrelin has a strong expression in the epithelial cells of the efferent ducts of the human epididymis, whereas there is no immunoreaction in its connective tissue (19). Moretti et al. (18) detected moderate level of ghrelin in DD and in few epithelial cells of the seminal vesicle in humans. In our study, ghrelin expression was limited by smooth muscle cells in the muscular layer of DD and smooth muscle cells in the media layer of the blood vessels in both bull and ram. In contrast to Moretti et al. (18) reports in humans, ghrelin expression was not found in epithelial cells of DD in bull and ram (Table 1).

According to recent studies, ghrelin has an effect on smooth muscle cells and accelerates gastric emptying (24). In humans, ghrelin has been described to be responsible for tonus of uterine smooth muscles and contractility (25). Qiu et al. (26) and Asakawa et al. (27) found that ghrelin administration in mice with experimental diabetes produced spontaneous increased contractions in smooth muscles by the activation of peripheral cholinergic pathways. In one study, ghrelin also affected different systems such as muscle motility and cell proliferation (28). Again, ghrelin stimulated and strengthened the contractions in the muscles in rats, mice, and guinea pigs (29). Deferent duct has a structure consisting of thick smooth muscles that makes powerful peristaltic movements during ejaculation, allowing the spermatozoa to flush out (30). In the present study, the expression of ghrelin that was found only in smooth muscle cells of DD, suggest that ghrelin may have the main role in stimulation of smooth muscle contractions during ejaculation in bulls and rams.

Although the testicular expression of obestatin in different cell types has been reported, the role of this peptide hormone in the male genital tract is still poorly understood (14). Dun et al. (31) and Moretti et al. (18) reported an intense expression of obestatin in DD, efferent duct and epithelial cells of seminal vesicle in rats and humans. In another study, obestatin was weakly expressed from apical cytoplasm of epithelial cells of seminal vesicle,

and it was not found in the connective tissue (23). As reported in the aforementioned studies (18, 23, 31), obestatin was positive in epithelial cells in DD and negative in stromal cells, and but it was released only from basal cells in bulls and rams. Previous studies have suggested that obestatin may play a role in cell proliferation, regulation of apoptosis and epithelial cell function. Our study suggests that intense expression of obestatin, especially in epithelial basal cells, may be associated with increasing epithelial proliferation and differentiation in these stem cell-treated cells.

In the 90s, the discovery of leptin was a major development about human metabolism, and it has been demonstrated that leptin has also played an effective role in the human reproduction (32). Leptin has direct effects on fertility in both male and female rodents, and it can reverse the infertility in ob / ob mice lacking the leptin gene (19). In some studies, a number of functions in male genital organs are regulated by leptin via the central nervous system. In recent years, it has been suggested that this hormone has a direct environmental effect on the target reproductive organs (22). In pigs, leptin expression was negative in immature genital duct epithelial cells and moderately positive in matures. In mice, leptin regulated the proliferation and differentiation of testicular germ cells (19). Studies in humans and laboratory animals have shown that leptin plays a key role by increasing muscle mass and myofibrilar hypertrophy in the musculature system (33). Leptin promotes proliferation in vascular smooth muscle cells by stimulating the transition from G1 phase to S phase during cell division, and ERK1 / 2 and NF-kB pathways support this process (34, 35). In our study, leptin expression was not found in the epithelium of DD, and it was found especially in the media layers of blood vessels and smooth muscle cells. This suggests that leptin increases muscle mass and hypertrophy in smooth muscle cells of DD in bull and ram. Besides, expressions in blood vessels also supports that this peptide hormone may play a role in the proliferation of vascular smooth muscle cells. Based on our results we can suggest that leptin has also a role in physiology of stromal cells in DD.

In mice and rats, leptin and ghrelin are expressed in endothelial and smooth muscle cells of blood vessels, but vascular effects of these two peptide hormones are not clear. The authors have stated that ghrelin and leptin may function in the regulation of vasodilatation, vasoconstriction or vascular permeability in blood vessels (36, 37). In the present study, we determined that ghrelin and leptin were expressed in the blood vessels localized in DD of bulls and rams.

In conclusion, ghrelin, obestatin and leptin were expressed in varying concentrations in epithelial, stromal and smooth muscle cells of the bull and ram DD. We can suggest that these hormones function in the contraction and hypertrophy of smooth muscles, and maybe in proliferation or apoptosis of epithelial and stromal cells in DD, too. However, we believe that we need more data to understand better the mechanisms that regulate the expressions and functions of ghrelin, obestatin and leptin in different layers of DD.

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Contributors: HS planned the study, designed the experiments and wrote the manuscript; BGS helped with data analyses, bioinformatics and manuscript writing; MEA, SE, UT and BB collected samples and conducted laboratory process; MAK analyzed the statistical data. All authors read and approved the final manuscript.

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IZRAŽANJE IN BIOLOŠKA AKTIVNOST GRELINA, OBESTATINA IN LEPTINA V SEMENOVODU PRI BIKU IN OVNU

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Povzetek: Znano je, da so grelin, obestatin in leptin vključeni v številne biološke procese, kot so uravnavanje apetita, sproščanje rastnega hormona in spodbujanje delitev celic. Poleg omenjenih bioloških učinkov pri ljudeh in glodavcih so v zadnjih letih postali znani tudi njihovi neposredni učinki na delovanje spolnega sistema in uravnavanje kontrakcije gladkih mišic. Pri sesalcih je delovanje spolnih žlez uravnano z zapleteno mrežo avtokrinih, parakrinih in endokrinih sporočil. Sporočila, ki sodelujejo pri nadzoru energetskega ravnotežja v telesu pogosto vplivajo tudi na delovanje spolnega sistema z vplivom na hipotalamusno-hipofizno-spolno os. Osnovni molekularni mehanizem delovanja spolnih žlez je slabo raziskan, na njegovo delovanje pa vplivajo genetski, okoljski in hormonski dejavniki. Grelin, obestatin in leptin so trije hormoni, ki v telesu sodelujejo pri urejanju in ohranjanju energetskega ravnotežja. Namen raziskave je bil preučiti imunohistokemično izražanje in potencialno biološko aktivnost grelina, obestatina in leptina v semenovodu bikov in ovnov. Grelin, obestatin in leptin so izraženi v semenovodu in so bili omejeni na citoplazmo epitelijskih in mišičnih celic. Grelin in leptin sta bila izražena v gladkih mišičnih celicah mišične plasti semenovoda in gladkih mišičnih celicah krvnih žil. Obestatin je bil izražen v bazalnih epitelijskih celicah svetline semenovoda. Grelin, obestatin in leptin so torej izraženi v različnih koncentracijah v epitelnih in mišičnih celicah ter krvnih žilah v semenovodu pri biku in ovnu. Glede na raznolike biološke vloge teh peptidnih hormonov lahko predvidevamo, da ti hormoni morda sodelujejo pri krčenju in hipertrofiji gladkih mišic ter pri množenju ali apoptozi epitelijskih in mišičnih celic v semenovodu.

Ključne besede: semenovod; grelin; leptin; obestatin; bik; oven; fiziološka funkcija

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Original Research Article

Salmonella Typhimurium BETWEEN 2000 AND 2012: ANTIMICROBIAL RESISTANCE AND PFGE PATTERNS OF ISOLATES FROM ANIMALS, HUMANS AND FOOD

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Abstract: *Salmonella* Typhimurium is an important zoonotic pathogen with high levels of antimicrobial resistance. In the present study, we compared the pulsed-field gel electrophoresis (PFGE) and antimicrobial resistance patterns of 275 *S.* Typhimurium isolates collected between 2000 and 2012: 93 from humans, 111 from animals and 71 from food. A high rate of antimicrobial resistance was detected (71.6%). Multidrug resistance (MDR), defined as phenotypic resistance to three or more antimicrobial classes, was detected in more than half of the isolates (54.9%). The proportion of MDR isolates was the highest in animals (43%), followed by food (30.5%) and humans (26.5%). Among 27 phenotypically determined resistance patterns, three were found to be most common: ACNaSuT (19.3%), ACSuT (12%) and ASuT (11.3%). The first two patterns were the most prevalent in animal isolates (47.2% and 51.5%, respectively), while ASuT isolates were most commonly obtained from humans (58.1%). Macrorestriction with *Xba*I revealed 72 pulsotypes in nine clusters (A to I) and 19 unique pulsotypes (X1-X19). By far largest cluster F with 29 pulsotypes comprised 58.2% of tested isolates and included 53.8% animal, 26.3% food and 20% human isolates. Two thirds (66.3%) of the isolates in this cluster were MDR. The second largest was cluster E with 11.4% isolates of 12 pulsotypes, including 59.4% human, 31.3% food and 9.4% animal isolates. MDR was detected in 78.1% isolates with the most prevalent resistance pattern ASuT. Cluster I (16 isolates, 2 genotypes) consisted of 87.5% human and 12.5% animal isolates. The majority of these isolates (81.3%) were sensitive to tested antimicrobials and MDR isolates (12.5%) were of ASuT resistance pattern. This work provided valuable data about resistance and PFGE patterns of *S.* Typhimurium isolates in Slovenia and as global knowledge is essential for improved surveillance of the infections, the data obtained could serve as a base for both national and multistate outbreak investigations.

Key words: *Salmonella* Typhimurium; human; animal; food; PFGE; antimicrobial resistance

Introduction

Salmonella can colonize a variety of animals. As the microbe is excreted via faeces and can survive in the environment, the latter may serve as the source of human and animal infections. Salmonellosis is the second most frequent food-borne zoonosis in the European Union (EU) (1). The main sources of human infections are poultry meat, eggs and dairy products (2). One of the most important nontyphoidal *Salmonella* serovars, beside the most prevalent *Salmonella* Enteritidis, is *Salmonella* Typhimurium (*S.* Typhimurium), which was in 2015 detected in 15.8% humans, 56.9% pigs, 43.2% cows and 5.8% poultry in the EU (1). *S.* Typhimurium-related salmonellosis is a major public health problem, especially because of the microbe's high resistance to antimicrobials. Although human salmonellosis often occurs as a self-limiting disease with diarrhoea, abdominal cramps and fever, the risk groups (infants, elderly people and immunocompromised patients) may be endangered by life-threatening infections, for

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which effective antimicrobials are needed (3). Antimicrobial resistance varies among *Salmonella* serovars countries. In general, *S.* Typhimurium shows a higher level of resistance than *S.* Enteritidis. As reported on the EU level for 2015, only 38.5% *S.* Typhimurium isolates in humans were susceptible to all tested antimicrobials and 44.4% of isolates were multidrug resistant (MDR) (4). Resistance to ampicillin, sulphonamides and tetracyclines was frequently detected, while resistance to third-generation cephalosporins is rarely found in nontyphoidal *Salmonella* serovars. Meanwhile, resistance to clinically important fluoroquinolones varies between serovars from humans and animals in different countries. Among the EU Member States (MSs), Slovenia showed the highest proportion of human *S.* Typhimurium isolates resistant to ciprofloxacin (41.7%). Furthermore, monophasic variant of *S.* Typhimurium 4,[5],12:i:- is associated with an increased resistance with 81.1% MDR (4).

In Slovenia, *S.* Typhimurium was the fourth most common serovar isolated from humans (9.9%) in 2015 (5), while the prevalence of *S.* Typhimurium in pigs was 9%. In poultry, the prevalence in 2015 was below 1%, with only one positive broiler flock (6). The presence of *S.* Typhimurium in cattle is currently not monitored.

Molecular tools such as pulsed-field gel electrophoresis (PFGE) are indispensable for disease surveillance, detection of outbreaks and epidemiological investigations. PFGE is a standardized method based on restriction of genomic DNA and is currently considered the gold standard for subtyping of different bacterial foodborne pathogens.

The aim of our study was to characterize *S.* Typhimurium isolates from humans, animals and food collected within 12 years by means of susceptibility testing and PFGE and to compare the characteristics between the groups of isolates in order to elucidate their potential relationships.

Materials and methods

Bacterial isolates

S. Typhimurium isolates (n=275) from humans (n=93), animals (n=111) and food (n=71) in the period between 2000 and 2012 were included in this study (Table 1). Human isolates from clinical salmonellosis cases were obtained from four regional offices of the National Laboratory of Health, Environment and Food and the National Institute of Public Health (NIPH). Animal isolates from the Institute of Microbiology and Parasitology of the Veterinary Faculty (IMP-VF) collection originated from clinically ill animals and from routine *Salmonella* monitoring samples. Food isolates were from the IMP-VF and NIPH collections. Isolates were stored in stab tubes and inoculated into blood agar before typing. All isolates were additionally confirmed as *Salmonella* sp. with MALDI-TOF (Bruker, Germany).

Antimicrobial susceptibility testing

The antimicrobial susceptibility was performed by determining the minimum inhibitory concentration (MIC) by microdilution method using commercially available microplate (EUVSEC, Sensititre®, Trek Diagnostic Systems, Thermo Fisher Scientific, USA). All isolates were phenotypically tested for their susceptibility to 14 antimicrobials from nine different antimicrobial classes: ampicillin (A), cefotaxime (Fot), ceftazidime (Taz), meropenem (Mero), nalidixic acid (Na), ciprofloxacin (Cip), tetracycline (T), colistin (Col), gentamicin (G), trimethoprim (Tm), sulfamethoxazole (Su), chloramphenicol (C), azythromicin (Azi) and tigecycline (Tig). *Escherichia coli* ATCC 25922 was used as a test control strain. The results were interpreted according to the European Committee on Antibiotic Susceptibility Testing (EUCAST) epidemiological cut-offs (7) and the recommendations of the European Union Reference Laboratory for Antimicrobial Resistance (EURL AMR) (8). The interpretative criteria were in concordance with the Decision 2013/652/EU of the European Commission (4). Multidrug resistance (MDR) was defined as phenotypic resistance to three or more antimicrobial classes.

PFGE

PFGE was carried out according to the standardised PulseNet protocol (9). Restriction patterns were analysed with BioNumerics software (v. 6.6, Applied Maths, Belgium). The relation between two isolates was scored using the Dice coefficient of similarity. Cluster analysis was performed by the unweighted pair-group

method with arithmetic means (UPGMA). Position tolerance and optimisation were set at 1.5% and 1%, respectively. Bands of size less than 33.3 kb were excluded from the analysis. Isolates with PFGE profiles of >88% similarity were considered to belong to the same cluster (marked by letters). Within a cluster, profiles differing from each other in at least one band were considered as subtypes (marked by digits).

Results

Overall, less than one third (28.4%, 78/275) of isolates were susceptible to all tested antimicrobials. Susceptibility was more frequently encountered in isolates from humans than in isolates from animals and food (51.3% vs. 38.5% and 10.3% isolates, respectively). Among resistant isolates (71.6%, 197/275), penta-resistance was most frequently encountered (22.5%), followed by

Table 1: Background of *Salmonella* Typhimurium isolates studied

Isolate origin	Category	No. of isolates (n=275)	Year of isolation
humans	*Salmonella* Typhimurium-infected patients	93	2000-2012
animals	pigs	47	2000-2002, 2005-2010
	poultry	39	2000-2011
	cows	3	2005, 2007
	other (not used for human consumption)	22	2000-2001, 2004, 2006, 2008-2012
food	pork meat	19	2002, 2005-2009, 2011
	poultry meat	25	2001-2007, 2009-2011
	beef	2	2006, 2008
	rabbit	1	2005
	minced meat (pork+beef)	24	2002-2009, 2012

Table 2: Overview of susceptibility testing results of 275 *Salmonella* Typhimurium isolates from animals, humans and food

Resistance to No. of antimicrobial classes	Total	Humans	Animals	Food
	No. (%)	No. (%)	No. (%)	No. (%)
6	10 (3.6)	0 (0.0)	9 (8.1)	1 (1.4)
5	62 (22.5)	17 (18.3)	30 (27.0)	15 (21.1)
MDR ≥5	***72 (26.2)***	*17 (18.3)*	*39 (35,1)*	*16 (22.5)*
4	36 (13.1)	4 (4.3)	18 (16.2)	14 (19.7)
3	43 (15.6)	19 (20.4)	8 (7.2)	16 (22.5)
MDR	***151 (54.9)***	*40 (43.0)*	*65 (58.6)*	*46 (64.8)*
2	18 (6.5)	6 (6.5)	8 (7.2)	4 (5.6)
1	28 (10.2)	7 (7.5)	8 (7.2)	13 (18.3)
Susceptible	78 (28.4)	40 (43.0)	30 (27.0)	8 (11.3)
Total	275	93	111	71

Table 3: Resistance patterns and PFGE clusters of 275 *Salmonella* Typhimurim isolates from humans, animals and food

Resistance to No. of antimicrobial clases	Resistance pattern	Cluster	No. (%)	Humans No. (%)	Animals No. (%)	Food No. (%)
6	ACGNaSuT	F	**6 (2.2)**	-	6 (100.0)	-
6	ACGNaSuTTm	F, X[a]	**4 (1.5)**	-	3 (75.0)	1 (25.0)
5	ACGSuT	F, E	**2 (0.7)**	1 (50.0)	-	1 (50.0)
5	**ACNaSuT**	**F**	**53 (19.3)**	**15 (28.3)**	**25 (47.2)**	**13 (24.5)**
5	ACNaSuTTm	F	**7 (2.5)**	1 (14.3)	5 (71.4)	1 (14.3)
≥5	*MDR ≥5*	*E, F, X*	*72 (26.2)*	*17 (23.6)*	*39 (54.2)*	*16 (22.2)*
4	ACSuTTm	F	**1 (0.4)**	1 (100.0)	-	-
4	ANaSuT	F	**1 (0.4)**	-	-	1 (100.0)
4	CGNaSuTm	F	**1 (0.4)**	-	1 (100.0)	-
4	**ACSuT**	**F, G, X[a]**	**33 (12.0)**	**3 (9.1)**	**17 (51.5)**	**13 (39.4)**
3	ACSu	F	**1 (0.4)**	-	1 (100.0)	-
3	AGT	E	**1 (0.4)**	-	1 (100.0)	-
3	ANaSu	F	**1 (0.4)**	-	-	1 (100.0)
3	ANaSuTm	E	**1 (0.4)**	-	-	1 (100.0)
3	**ASuT**	**E, C, D, I, X[a]**	**31 (11.3)**	**18 (58.1)**	**3 (9.7)**	**10 (32.3)**
3	ASuTTm	A, X[a]	**4 (1.5)**	1 (25.0)	-	3 (75.0)
3	ATTm	B	**1 (0.4)**	-	-	1 (100.0)
3	GNaSu	F	**2 (0.7)**	-	2 (100.0)	-
3	GSuT	F	**1 (0.4)**	-	1 (100.0)	-
≥3	*MDR*	*A, B, C, D, E, F, G, I*	*151 (54.9)*	*40 (26.5)*	*65 (43.0)*	*46 (30.5)*
2	ASu	E, F, G, X[a]	**6 (2.2)**	3 (50.0)	1 (16.7)	2 (33.3)
2	AT	X[a]	**1 (0.4)**	-	-	1 (100.0)
2	GSu	F	**1 (0.4)**	-	1 (100.0)	-
2	NaSu	F	**7 (2.5)**	1 (14.3)	6 (85.7)	
2	SuT	H, G	**3 (1.1)**	2 (66.7)	-	1 (33.3)
1	G	G	**3 (1.1)**	2 (66.7)	1 (33.3)	-
1	Na	F, X[a]	**4 (1.5)**	-	1 (25.0)	3 (75.0)
1	Su	F, I	**15 (5.5)**	1 (6.7)	5 (33.3)	9 (60.0)
1	T	C, A, G, E, H	**6 (2.2)**	4 (66.7)	1 (16.7)	1 (16.7)
0	susceptible	F, G, I, H, E, B, X[a]	**78 (28.4)**	40 (51.3)	30 (38.5)	8 (10.3)
	Total		**275**	93 (33.8)	111 (40.4)	71 (25.8)

Table 4: Distribution of MICs and resistance of 275 *Salmonella* Typhimurium isolates from animals, humans and food, collected between 2000 and 2012

Antimicrobial		Resistance No.	MIC distribution (mg/l) - number and percent of isolates																		Range	MIC_{50}	MIC_{90}
		%	0,008	0.015	0.03	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024			
Ampicillin	A	154								70	48	3				1	153					1 - >64	>64	>64
		56,0								25,5	17,5	1,1				0,4	55,6							
Cefotaxime	Fot	0						266	9													0,25 - 0,5	>0,25	>0,25
		0,0						96,7	3,3															
Ceftazidime	Taz	0							271	4												0,5 - 1	>0,5	>0,5
		0,0							98,5	1,5														
Meropenem	Mero	0			259	16																0,03 - 0,064	>0,03	>0,03
		0,0			94,2	5,8																		
Nalidixic acid	Na	87										179	8	1		1	18	68				4 - >128	>4	>128
		31,6										65,1	2,9	0,4		0,4	6,5	24,7						
Ciprofloxsacin	Cip	87		151	37		39	44	3	1												0,015 - 1	>0,015	>0,25
		31,6		54,9	13,5		14,2	16,0	1,1	0,4														
Tetracycline	T	155									117	3		2	48	54	51					2 - >64	>32	>64
		56,4									42,5	17,6		0,7	17,5	19,6	18,5							
Colistin	Col	0								240	35											1 - 2	>1	>2
		0,0								87,3	12,7													
Gentamicin	G	21							240	12	2	2	2	2	5	10						0,5 - >32	>0,5	>1
		7,6							87,3	4,4	0,7	0,7	0,7	0,7	1,8	3,6								
Trimethoprim	Tm	19						234	18	4						19						0,25 - >32	>0,25	>0,5
		6,9						85,1	6,5	1,5					0,0	6,9								
Sulfamethoxazole	Su	181											21	23	36	11	3				181	8 - >1024	>1024	>1024
		65,8											7,6	8,4	13,1	4,0	1,1				65,8			
Chloramphenicol	C	108											166	1	1	3	39	65				8 - >128	>8	>128
		39,3											60,4	0,4	0,4	1,1	14,2	23,6						
Azithromycin	Azi	0,0									115	141	15	4								2 - 16	>4	>4
		0,0									41,8	51,3	5,5	1,5										
Tigecycline	Tig	0						168	97	10												0,25 - 1	>0,25	>0,5
		0,0						61,1	35,3	3,6														

resistance to three (15.6%), four (13.1%) and one (10.2%) class of antimicrobials. Resistance to two and six classes was less common, seen in 6.5% and 3.6% of isolates. More than half of all isolates (54.9%, 151/275) were determined as MDR. The proportion of all MDR isolates was the highest in animals (43%), followed by food (30.5%) and humans (26.5%). Among animal isolates, MDR was mostly found in isolates from farm animals (68.5% in farm animals vs. 18.2% in non-farm animals, data not shown). Detailed information about susceptibility testing results is shown in Table 2.

Among 27 phenotypically determined resistance patterns, three were found to be most common: ACNaSuT (19.3%, 53/275), ACSuT (12%, 33/275) and ASuT (11.3%, 31/275), while the remaining patterns were detected in lower proportions (range 0.4% to 5.5%). Resistance patterns ACNaSuT and ACSuT were the most prevalent in animal isolates (47.2% and 51.5%, respectively), while ASuT isolates were most commonly obtained from humans (58.1%).

Correlation between resistance patterns and genotypes was not evident. The majority of resistance patterns (i.e. 19) were related to only one PFGE cluster, while seven resistance patterns were found in two or more clusters. The most prevalent resistance pattern ACNaSuT was confined to cluster F, ACSuT isolates were found in clusters F and G and ASuT isolates in four clusters (E, C, D and I). The findings related to resistance patterns are summarized in Table 3, while MIC distribution is shown in Table 4.

Macrorestriction with *Xba*I revealed 72 pulsotypes in nine clusters (A to I) and 19 unique pulsotypes (X1-X19). Human isolates exhibited 31 distinct pulsotypes grouped in seven clusters and seven unique pulsotypes. In animal isolates, 34 different pulsotypes were distributed into seven clusters; in addition, nine unique pulsotypes were identified. Among food isolates, three non-clustered pulsotypes were detected, while 25 pulsotypes grouped in eight clusters. An overview of PFGE clusters and pulsotypes is given in Table 5.

By far the largest cluster F with 29 pulsotypes comprised 58.2% (160/275) of tested isolates and included 53.8% animal, 26.3% food and 20% human isolates. Two thirds (66.3%) of the isolates in this cluster were MDR. The most common pulsotype in this cluster was F9, encompassing 51.3% (82/160) of isolates in the cluster (data not shown). Isolates with F9 pulsotype were of animal (46.3%), food (34.1%) and human (19.5%) origin. About three quarters (76.8%) of F9 isolates were MDR. The two most common resistance patterns of F9 isolates were ACNaSuT and ACSuT. The former was detected only in cluster F and the latter was identified in a vast proportion (87.9%) in the same cluster.

The second largest was cluster E with 32/275 (11.4%) isolates of 12 pulsotypes, including 59.4% human, 31.3% food and 9.4% animal isolates. MDR was detected in 78.1% isolates with the most prevalent resistance pattern ASuT.

Cluster I (16 isolates, 2 genotypes) consisted of 87.5% human and 12.5% animal isolates.

Table 5: Distribution of PFGE clusters among 275 *Salmonella* Typhimurim isolates from humans, animals and food

Cluster	No. of PFGE patterns	Isolates No. (% of total)	Humans No. (% of cluster)	Animals No. (% of cluster)	Food No. (% of cluster)
A	4	4 (1.5)	1 (25.0)	0 (0.0)	3 (75.0)
B	4	4 (1.5)	0 (0.0)	1 (25.0)	3 (75.0)
C	3	6 (2.2)	4 (66.7)	1 16.7)	1 (16.7)
D	1	2 (0.7)	0 (0.0)	0 (0.0)	2 (100.0)
E	12	32 (11.6)	19 (59.4)	3 (9.4)	10 (31.3)
F	29	160 (58.2)	32 (20.0)	86 (53.8)	42 (26.3)
G	10	20 (7.3)	12 (60.0)	5 (25.0)	3 (15.0)
H	7	12 (4.4)	4 (33.3)	4 (33.3)	4 (33.3)
I	2	16 (5.8)	14 (87.5)	2 (12.5)	0 (0.0)
X[a]	19	19 (6.9)	7 (36.8)	9 (47.4)	3 (15.8)
Total	91	275	93	111	71

[a] unique PFGE patterns

The majority of isolates (81.3%) were sensitive to tested antimicrobials, MDR isolates (12.5%) were of ASuT resistance pattern.

The remaining clusters were smaller, comprising 2 to 20 isolates and 2 to 10 genotypes (Table 5).

Discussion

S. Typhimurium isolates investigated in the present study exhibited a high level of antimicrobial resistance as only 28.4% isolates were susceptible to all antimicrobials tested. *S.* Typhimurium and monophasic *S.* Typhimurium are reported to contribute significantly to the overall numbers of MDR *Salmonella* in Europe (4). More than half of *S.* Typhimurium isolates from humans in the EU were resistant to ampicillin (56.3%), sulfonamides (52.4%) and tetracycline (51.9%) with high to extremely high levels in most reporting MSs. Even higher proportions of resistance were observed in monophasic *S.* Typhimurium from humans, where close to 90% of all isolates were resistant to these three antimicrobials. The proportions of isolates resistant to either of the two clinically most critical antimicrobials were rare (on average 6.6% for ciprofloxacin and 1.1% for cefotaxime). This is in concordance with the findings of the present study even though it is challenging to compare the results of resistance to ampicillin, sulfonamides and tetracycline for human isolates due to low numbers of tested isolates per year (range 1 to 19). When more than 10 isolates were tested within the same year, the resistance levels ranged from 31.6% to 55.6% for sulfonamides, 26.3% to 50% for tetracycline and 26.3% to 55.6% for ampicillin (data not shown). Resistance to cefotaxime, ceftazidime, meropenem, colistin, azithromycin and tigecycline was not recorded. A total of ten monophasic *S.* Typhimurium isolates were tested; three from humans, two from animals and five from food (data not shown). All isolates with the exception of one from food were MDR. All isolates were resistant to ampicillin and sulfamethoxazole and nine of them also to tetracycline.

Slovenia was ranked fourth among the MSs with extremely high and very high *S.* Typhimurium MDR with 55.3% MDR human isolates in 2015 (4). In addition, the temporal trend analysis performed for the 3 years 2013–2015 showed statistically significant increases in (fluoro)quinolone resistance in Slovenia. Furthermore, the highest proportion of isolates resistant to ciprofloxacin was reported from Slovenia (41.7%), but the number of isolates tested was low (n = 48). Reduced susceptibility of human isolates to ciprofloxacin is of special concern in Italy where 91.7% of isolates had MIC ≥ 0.125 mg/l, which is the cut-off value according EUCAST (10). Resistance to ampicillin and tetracycline also increased significantly in Slovenia (4). As mentioned before, differences in number of tested isolates per year in the present study render relevant comparison impossible, but the overall (2000-2012) MDR in human isolates was found to be 43% (Table 2), with 18.3% isolates being resistant to five classes of antimicrobials. This is comparable to the data in the EU in 2015 when MDR was reported to be 44.4% in *S.* Typhimurium and 81.1% in monophasic *S.* Typhimurium. Half of the MSs testing isolates for the nine antimicrobial classes included in the MDR analysis reported a few isolates resistant to at least six of the classes (4).

The resistance levels of human and animal *S.* Typhimurium isolates are even more concerning in China as 84.6% of tested isolates were MDR with 22 isolates resistant also to cephalosporines and ciprofloxacin. Additionally, six isolates were also resistant to azithromycin, which is the drug of choice for invasive infections (11, 12). Another study reported about an isolate from retail market, which was resistant to ten antimicrobials. This may represent substantial risk to public health in the future due to food industry globalization and the fact that resistance genes can be easely disseminated between bacteria (13).

The number of MDR *S.* Typhimurium isolates from human varies between countries; from only 11% in Finland, 29% in USA, 43.9% in Belgium to 86.8% in China (14, 15, 16, 17). These studies reported ACSSuT resistance pattern as the most prevalent in humans which contrasts the results of the present study where only 9.1% of human isolates showed ACSuT pattern (susceptibility to streptomycin was not tested). The most common MDR pattern in human isolates found in the present study was ASuT (58.1%), followed by ACNaSuT (28.3%).

Antimicrobials such as ampicillin, sulfamethoxazole and tetracycline have been widely used for many years in veterinary medicine to treat infections in production animals. Generally, MSs reported high levels of resistance to these antimicrobials from producing animals and meat products thereof. Overall, very high resistance to

ampicillin, sulfamethoxazole and tetracycline was observed in *S.* Typhimurium isolates from carcases of fattening pigs in the EU; 52.4% isolates were MDR and the most frequent MDR core pattern was ASuT. According to the data from the present study, ASuT resistance pattern was the third most prevalent among the studied isolates, being shown by 11.3% isolates. However, 58.1% of ASuT isolates were of human origin in contrast to only 9.7% of animal origin. But this pattern, along with the additional resistance to chloramphenicol and nalidixic acid, was the most prevalent in isolates studied (19.3%), especially in animals (47.2%). In pigs, 76.6% isolates were MDR, 29.8% of them showing ACNaSuT resistance pattern. For comparison, in Belgium only 31.2% pig isolates showed MDR pattern and as little as 3.4% isolates were resistant to nalidixic acid, which was in concordance with MDR prevalence in pork (16). Even better situation was reported in Sweden, where only 11% of animal isolates were MDR with the most common resistance pattern ACSSuT (18). In poultry, a slightly lower proportion of MDR compared to pigs was detected in the present study (59%) with the most prevalent resistance pattern being ACSuT. Resistance to nalidixic acid, which is an indicator agent for fluoroquinolone resistance prediction, was detected in 33.3% poultry isolates in contrast to 70.2% in pig isolates (data not shown). A high prevalence of nalidixic acid resistance was expected for isolates from pork meat, but it was only deteceted in 31.6%. Data about the origin of the pork meat could perhaps clarify the findings, as lower prevalence of nalidixic acid resistance could linked to imported pork meat. On the contrary, the proportions of nalidixic acid resistant isolates from poultry meat (32%) and beef meat (50%) were comparable with the proportions detected in isolates from poultry (33.3%) and cattle (66.7%). Similarly, a difference between pig and pork meat isolates was observed for chloramphenicol resistance (63.8% vs. 31.6%), while the proportions were comparable for other tested antimicrobials (data not shown). A notable difference in the number of MDR isolates was observed between production and non-production animals, as only 18.2% non-production animals were MDR compared to 68.5% farm animals (data not shown). This suggests that the food animal production technology should probably implement measures to prevent the outbreaks of the diseases, to stop the spread of bacterial pathogens and apply consistent laboratory diagnostics, which could lead to the reduction of antibiotic use and empiric treatment of the diseases. Resistance to five or more antimicrobial classes was seen almost exclusively in isolates from production animals with the exception of one isolate from a mouse which was caught on a poultry farm. Isolate from the mouse shared PFGE (genotype F9) and resistance characteristics (ACNaSuT) with isolates from poultry in the same time period which may be either a coincidence or may indicate mice as vectors of *S.* Typhimurium on farms. Furthermore, the same resistance pattern and genotype as in mouse was also detected in poultry meat and in humans in 2011.

PFGE analysis provided an insight into genetic diversity of isolates; a total of 91 PFGE patterns among 275 isolates were defined. Reports on *S.* Typhimurium diversity vary; Rounds et al. (19) described the serovar to be of low clonality in congruence with a few studies, while in several other reports the isolates exhibited high genetic similarity (20). Nineteen out of 91 patterns in the present study were unique and 40.7% of isolates grouped into three clusters (E, F and G). For comparison, *S.* Infantis isolates in Slovenia were found to be more genetically homogeneous as 74.7% of isolates grouped in only two clusters (data not published). PFGE has been used for epidemiological investigations to study *S.* Typhimurium PFGE patterns in the food chain and it has been demonstrated that genetically similar isolates were transferred along the slaughterhouse line and to retail markets (13). Identical PFGE patterns (e.g. F1, F7, F9) from animals, food and humans have been identified also in the present study and five of a total of nine *S.* Typhimurium clusters included isolates of all three origins. However, unfortunately there were no evident epidemiological links revealed as the isolates analysed were geographically and temporally scattered. Interestingly, the largest cluster F contained 58.2% of analysed isolates and 51.3% among them were characterized by F9 pattern which seems to be widespread, perhaps due to its temporal and genetic stability as described before (17). By comprising 77.5% of all animal isolates in the study, cluster F was mainly linked to animal isolates. Overall, 66.3% isolates in this cluster were MDR with common resistance patterns ACSuT and ACNaSuT, which were found to be related mostly to poultry and

pigs, respectively. On the other hand, clusters E, G and I comprised mostly human isolates; overall MDR in these clusters was 41.2% and common resistance pattern was ASuT.

Discussion about the detected PFGE patterns among different studies is hampered by the fact that reliable comparisons can only be achieved by using common PFGE databases. Ongoing establishment of a joint database for foodborne pathogens on the EU level, following the example of PulseNet, will undoubtedly facilitate interlaboratory comparison of typing results and enhance the surveillance of foodborne pathogens in the food chain.

In conclusion, the number of *S.* Typhimurium isolates in humans and animals does not seem to change much over the years; however, because of the worrying resistance levels, monitoring of all categories of production animals should be implemented in every MS. Lienemann et al. (14) suggested that the source of MDR *S.* Typhimurium for humans in Finland could be imported food sold in supermarkets and restaurants as MDR *Salmonella* is rare among domestic animal production. Therefore, global knowledge about prevalence, resistance patterns and genetic characteristics is essential for successful control of outbreaks on the international level. This work, the first of its kind in Slovenia regarding *S.* Typhimurium, provided valuable data about resistance and PFGE patterns of *S.* Typhimurium isolates from animals, humans and food on the national level and the data obtained could serve as a base for both national and multistate outbreak investigations.

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Salmonella Typhimurium MED LETOMA 2000 IN 2012: VZORCI ODPORNOSTI PROTI PROTIMIKROBNIM ZDRAVILOM IN VZORCI PFGE IZOLATOV IZ ŽIVALI, LJUDI IN ŽIVIL

J. Mićunović, M. Pate, J. Avberšek, M. Ocepek

Povzetek: *Salmonella* Typhimurium *je* pomembna povzročiteljica zoonoz, pri katerih se pojavlja visok odstotek proti protimikrobnim zdravilom odpornih sevov. V raziskavi smo primerjali restrikcijske vzorce, pridobljene z metodo elektroforeze v pulzirajočem električnem polju (PFGE), in vzorce odpornosti 275 izolatov *S.* Typhimurium, izoliranih med letoma 2000 in 2012 iz 93 ljudi, 111 živali in 71 vzorcev hrane. Dokazali smo visok odstotek odpornih sevov (71,6 %). Odpornost proti trem ali več skupinam protimikrobnih zdravil (VOB) smo ugotovili pri več kot polovici izolatov (54,9 %). Največ VOB izolatov smo ugotovili pri živalih (43 %), sledita hrana (30,5 %) in ljudje (26,5 %). Med 27 fenotipskimi vzorci odpornosti so bili najpogostejši trije: ACNaSuT (19,3 %), ACSuT (12 %) in ASuT (11,3 %). Prva dva vzorca sta bila najpogostejša pri živalih (47,2 % in 51,5 %), medtem ko so bili izolati z vzorcem ASuT najpogosteje dokazani pri ljudeh (58,1 %). Na podlagi makrorestrikcije z encimom *Xba*I smo ugotovili 72 vzorcev PFGE, razvrščenih v devet genetskih skupin (A – I), in 19 edinstvenih vzorcev (X1 – X19). Največja je bila genetska skupina F z 29 vzorci, ki so predstavljali 58,2 % vseh izolatov (53,8 % iz živali, 26,3 % iz hrane in 20 % iz ljudi). Dve tretjini (66,3 %) izolatov v genetski skupini sta bili VOB. Druga največja je bila genetska skupina E z 11,4 % vseh izolatov (12 vzorcev PFGE), od tega 59,4 % iz ljudi, 31,3 % iz hrane in 9,4 % iz živali. VOB je bila dokazana pri 78,1 % izolatov z najpogostejšim vzorcem odpornosti ASuT. V genetski skupini I (16 izolatov, 2 genotipa) je bilo 87,5 % izolatov iz ljudi in 12,5 % izolatov iz živali. Večina teh izolatov (81,3 %) je bila občutljiva na vsa testirana protimikrobna zdravila, medtem ko so izolati VOB (12,5 %) imeli vzorec ASuT. V raziskavi smo pridobili dragocene podatke o odpornosti in vzorcih PFGE bakterije *S.* Typhimurium na nacionalni ravni. Za uspešen nadzor izbruhov je zelo pomembno globalno poznavanje te tematike, zato so podatki iz te raziskave pomemben prispevek k nacionalnemu in mednarodnemu preiskovanju izbruhov.

Ključne besede: *Salmonella* Typhimurium; človek; žival; hrana; PFGE; odpornost

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Original Research Article

HEMATO-BIOCHEMICAL AND HISTOPATHOLOGICAL CHANGES IN MUD LOACH, *Misgurnus mizolepis* EXPERIMENTALLY INFECTED WITH *Aeromonas sobria*

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Abstract: Hemato-biochemical and histopathological alterations in mud loach (*Misgurnus mizolepis*) infected with *Aeromonas sobria* were characterized in the present study. Fish infected with 1×10^6 colony forming units/mL of *A. sobria* (25 fish × 3 replicates × 3 tanks) and uninfected (control) fish (25 fish × 3 replicates × 3 tanks) were kept in laboratory tanks with adequate water parameters, and euthanized at 1, 3, and 5 days post-infection (PI). Infected fish became lethargic and developed rapid opercular movements, pale gills, and cutaneous petechial hemorrhages during the 5-day experimental period. Hemato-biochemical parameters, namely hematocrit, hemoglobin, total protein, total calcium, alkaline phosphatase (ALP) activity, and glucose level were significantly reduced, whereas urea (UA) level, and aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) activities were markedly increased in infected fish compared to control fish. Total leukocyte count was higher of infected than in control fish by day 3 PI, but dramatically decreased on day 5 PI. The leukogram showed the predominance of lymphocytes and neutrophils during the experimental period. Histologically, kidney and spleen showed extensive hemorrhage with cellular necrosis. Liver cells showed severely necrotic foci with karyopyknosis, karyorrhexis, karyolysis, and hyperchromatosis of the nuclear membrane. Thus, *A. sobria* infection causes hemato-biochemical changes such as anemia, hypoglycemia, hypoproteinemia, hypocalcemia, increased AST, ALT, and LDH activities, increased UA concentrations, and reduced ALP activity, and lead to compromised disease resistance, tissue damage, and low survival rates.

Key words: *Aeromonas sobria*; hematology; histopathology; *Misgurnus mizolepis*

Introduction

The mud loach, *Misgurnus mizolepis* (Cobitidae, Cypriniformes), is a freshwater fish that is widely distributed throughout streams, ditches, reservoirs, and rice paddy fields, preferably with a soft muddy bottom, in Korea, Japan, and China (1).

Aeromonas sobria has been isolated from human blood, feces, and wounds (2, 3, 4), and from wild-spawning gizzard shad, *Dorosoma cepedianum* (5), European perch, *Perca fluviatilis* (6), and mud loach (7). Moribund gizzard shad did not display any clinical signs of disease whereas European perch and mud loach displayed hemorrhage and ulceration on skin and fins.

Hematological parameters and histological changes have been widely used to assess the health status of fish exposed to various diseases, both in laboratory and field studies (8, 9, 10, 11, 12, 13).

A previous study on *A. sobria* infecting mud loach described the histopathology of several tissues, the etiology of morbidity and mortality, the characteristics of *A. sobria*, and confirmed

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bacterial toxins using polymerase chain reaction (PCR) (7). To our knowledge, the hematological changes occurring in mud loach infected with *A. sobria* have not been reported so far, although they could provide insight into fish responses to this bacterial infection, its disease process, and on the degree of lesion and metabolic disorder, all of which facilitate disease diagnosis. Thus, the present study investigated changes in clinical signs and evaluated blood chemistry and, tissue pathology in the mud loach, aiming to better characterize its diseased state.

Materials and methods

Fish

Three hundred fifty clinically healthy mud loach individuals (average body weight 6.6 g, two years old, both sexes) were collected from a holding fish farm located in Buan-gun, Jeollabuk-do, Korea, and transferred to the laboratory alive, within polyethylene bags containing oxygenated water. These fish were acclimatized in a plastic tank (1.5 tonnes) filled with dechlorinated tap water (pH 6.8-7.1; dissolved oxygen 6.0-6.5 mg/L) for 30 days. The tank was continuously aerated and the water temperature was maintained at 20 ± 1 °C. Approximately 50% of the water was replaced daily. Ammonia, nitrite, and pH levels were measured on alternate days using a water-testing kit (Hach, Loveland, CO, USA). Because mud loach is an air-breathing fish, the oxygen level in the tank was not measured. Fish were fed daily with commercial mud loach feed (Woosung Aquafeed, Daejeon, Korea) at 1% of their biomass until three days before infection. After acclimatization, 300 fish were divided into four groups of 75 fish each distributed across 12 tanks (40-L capacity), each containing 25 fish (experiments were run in triplicate). Before infection, fish in each tank were acclimatized for seven days but not fed, in order to standardize their dietary status. During this period, the water was maintained at 20 ± 1 °C, pH 6-7, dissolved oxygen above 6 mg/L, and nitrite below 1 mg/L. All procedures were approved by the Experimental Animal Ethical Committee of the Kunsan National University (Reg. No. 410000100008).

Experimental infection

Aeromonas sobria (LBH) used in this study was previously isolated from a naturally infected mud loach (7). Before the experimental infection, three consecutive serial passages of the bacteria in mud loach individuals anaesthetized with AQUI-S 10 (Handong Co., Seoul, Korea) in accordance with the manufacturer's manual were executed by the intraperitoneal injection of about 1×10^7 colony forming units (CFU) and re-isolation. After each passage, bacteria were suspended in a sterile saline solution (0.85% NaCl). Two treatments were applied: control fish (25 fish per tanks, three tanks, 75 fish in total) were injected with 0.1 mL of the sterile saline solution; infected fish (25 fish per tank, nine tanks, 225 fish in total) were injected with 1×10^6 CFU/mL of LBH intraperitoneally. During the experiment, the water (dechlorinated) in the experimental tanks was maintained at 20 ± 1°C, pH 6-7, dissolved oxygen above 6 mg/L, and nitrite below 1 mg/L. Fish were observed daily throughout the experimental period.

Hematological analysis

Blood samples were collected from 15 fish per tank at 1, 3, and 5 days after the onset of the experiment. Fish were captured with nets and quickly anaesthetized with AQUI-S 10 according to the manufacturer's manual. Blood was drawn from the caudal vein using 1-mL disposable syringes. Hematocrit (Ht) was measured as the volume of packed erythrocytes using a micro-hematocrit centrifuge (RPM12000, Hawksley, Sussex, UK), and hemoglobin (Hb) was determined by the cyanhemoglobin method. Total leukocyte count (TLC) was determined in a Neubauer chamber using Natt and Herrick's solution as the diluent. Differential leukocyte counts were carried out using blood smears stained with May-Grünwald Giemsa (13). The morphological features of differential leukocytes were determined according to Gao *et al.* (14). One hundred leukocytes were randomly selected from three slides per fish for measuring erythrocyte size (i.e., the length of the longest axis) in an oil immersion under the light microscope, using the measuring function of the imaging software (cellSens Entry, Olympus, Tokyo, Japan). Total protein (TP), total calcium (Ca), urea (UA) and glucose (GLU) concentrations,

as well as alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) activities in the plasma were measured using an automatic dry chemistry analyzer (DRI-CHEM 3500i, Fuji, Tokyo, Japan).

Histopathological analysis

Liver, spleen, and kidney tissues were aseptically collected from the same 15 fish used for blood sampling and hematological analysis. Those tissues were fixed in 10% neutral buffered formalin, dehydrated in a graded series of ethanol, and embedded in paraffin block preparation. Tissue sections were cut at 5 µm, mounted on slides, stained in hematoxylin and eosin (H&E), and observed under the light microscope and photographed for histopathological examination.

Statistical analysis

Results are expressed as means ± standard deviation (SD). Hematological data were analyzed in SPSS 7.5 for Windows using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test to determine significance differences among groups, considering $P < 0.05$.

Results

Clinical signs of disease and gross lesions

Mud loach began to show clinical signs of disease and morbidity two days after inoculation with *A. sobria*. At three to five days post-infection (PI), the skin of infected fish presented cutaneous petechial hemorrhages on caudal and pectoral fins and abdominal and lateral body (Figure 1A). Petechiae were also found on the mouth, isthmus, cheek, operculum (Figure 1B) and caudal and pectoral fins presented hyperemia at their bases (Figure 1C). Infected fish became lethargic and developed rapid opercular movements and pale gills before dying. Internally, infected fish exhibited liver enlargement on day 2 PI, and then became pale, softened, and presented punctuate hemorrhage over the next 24 h. Kidney and spleen of infected fish showed enlargement and anemia on days 2 to 5 PI. During the experimental period, cumulative mortality occurred at a rate of 13.7 % (31 of the 225 fish). No clinical sings, macroscopic lesions and mortality occurred in control fish.

Hemato-biochemical characteristics

Regarding erythrocyte parameters, Ht level of infected fish was lower than that of control fish on day 1 PI (Table 1), and Ht reductions tended to be more severe in infected groups on days 3 and 5 PI. The Hb level of infected fish was slightly lower than that of control fish, although this difference was not statistically significant on day 1 PI. The level was further reduced on days 3 and 5 PI. The TLC of infected fish was higher than that of control fish by day 3 PI, but it dramatically decreased on day 5 PI. Infected fish showed higher AST, ALT, and LDH activities and UA levels than control fish on day 1 PI, and these levels were considerably increased on days 3 and 5 PI. The levels of TP, ALP, Ca, and GLU were slightly reduced in infected fish compared to control fish at day 1 PI, but on days 3 and 5 PI, significant reductions were observed.

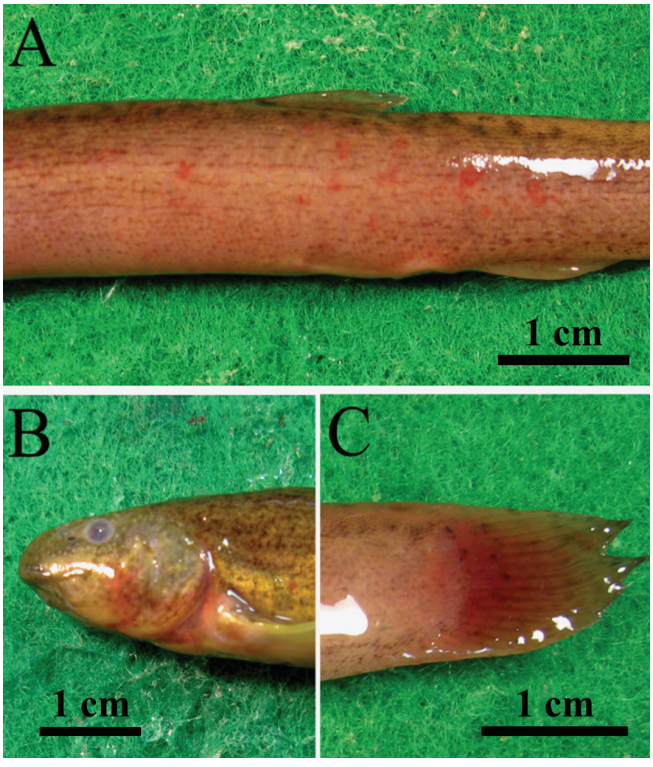

Figure 1: Hemato-biochemical characteristics of mud loach, *Misgurnus mizolepis*, experimentally infected with *Aeromonas sobria*. The mud loach displayed hemorrhage on the lateral portion of body (A), operculum, cheek (B), and fin base (C).

Table 1: Hemato-biochemical characteristics of mud loach, experimentally infected with *Aeromonas sobria*

Hemato-biochemical characteristics	Control	Days post-infection		
		Day 1	Day 3	Day 5
Hematocrit (%)	37.8 ± 1.2^{a}	30.7 ± 5.2^{a}	22.7 ± 2.2^{b**}	13.3 ± 3.0^{c**}
Hemoglobin (g/dL)	5.4 ± 0.5^{a}	5.0 ± 1.0^{a}	4.2 ± 2.2^{b}	2.8 ± 2.8^{c*}
Erythrocyte diameter (μm)	8.2 ± 0.3^{a}	8.2 ± 0.3^{a}	7.5 ± 0.6^{b}	6.9 ± 0.5^{c*}
TLC (10^3/mm^3)	80.5 ± 3.5^{a}	90.5 ± 5.6^{b*}	101.5 ± 6.0^{c**}	55.5 ± 3.5^{d}
Total Protein (g/L)	5.7 ± 0.6^{a}	4.6 ± 0.2^{b}	3.9 ± 0.5^{c**}	2.8 ± 0.7^{d**}
ALP (KA)	8.0 ± 1.2^{a}	7.7 ± 0.7^{a}	5.9 ± 0.6^{b**}	4.0 ± 1.7^{b**}
Total Calcium (mg/dL)	10.8 ± 0.3^{a}	10.9 ± 1.5^{a}	8.4 ± 1.3^{b*}	6.3 ± 1.0^{c**}
Urea (mg/dL)	5.8 ± 0.4^{a}	6.6 ± 2.0^{ab}	9.3 ± 1.6^{c**}	13.8 ± 2.1^{d**}
AST (U/L)	109.3 ± 22.8^{a}	145.0 ± 14.6^{b**}	200.5 ± 28.1^{c**}	312.0 ± 35.0^{d**}
ALT (U/L)	22.5 ± 6.8^{a}	38.7 ± 5.0^{b*}	56.0 ± 6.3^{c**}	79.0 ± 10.8^{d**}
LDH (U/L)	322.9 ± 36.6^{a}	459.3 ± 68.5^{b*}	727.8 ± 60.2^{c**}	822.8 ± 59.9^{d**}
Glucose (mg/dL)	195.7 ± 24.4^{a}	198.8 ± 18.9^{a}	115.3 ± 21.9^{b**}	80.5± 14.5^{c**}

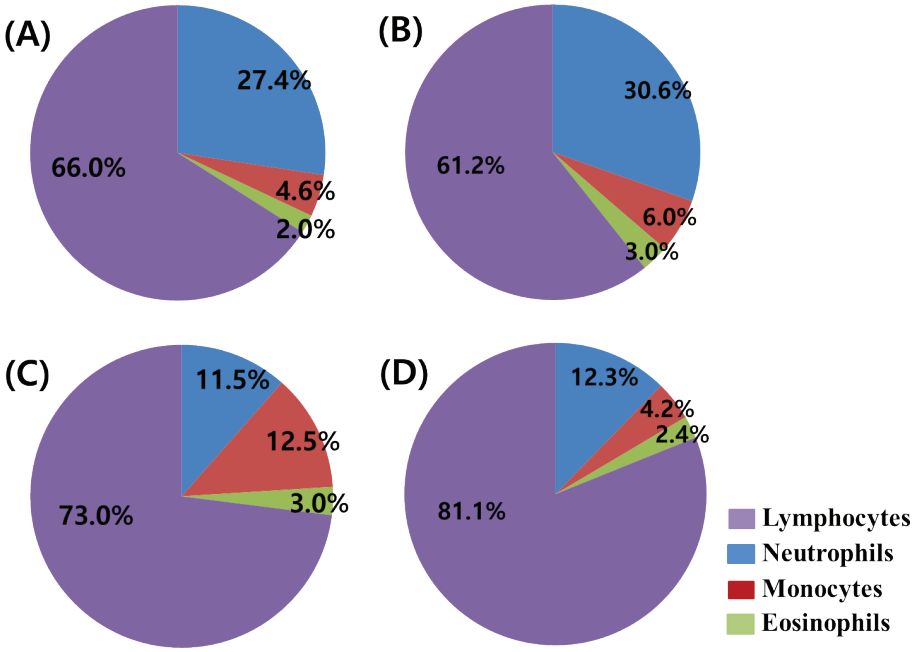

Figure 2: Leukograms of mud loach, *Misgurnus mizolepis*, in the control group (A), and at days 1 (B), 3 (C), and 5 (D) post-infection with *Aeromonas sobria*.

Table 2: Frequency (%) of erythrocytes in each diameter range in mud loach experimentally infected with *Aeromonas sobria*

Range of erythrocytes diameter (µm)	Control	Days post-infection		
		Day 1	Day 3	Day 5
5.5-6.0			1	4
6.1-6.5			4	18
6.6-7.0			15	39
7.1-7.5	1		29	25
7.6-8.0	39	38	38	13
8.1-8.5	48	49	9	1
8.6-9.0	11	12	4	
9.1-9.5	1	1		
Number of fish examined	15	15	15	15

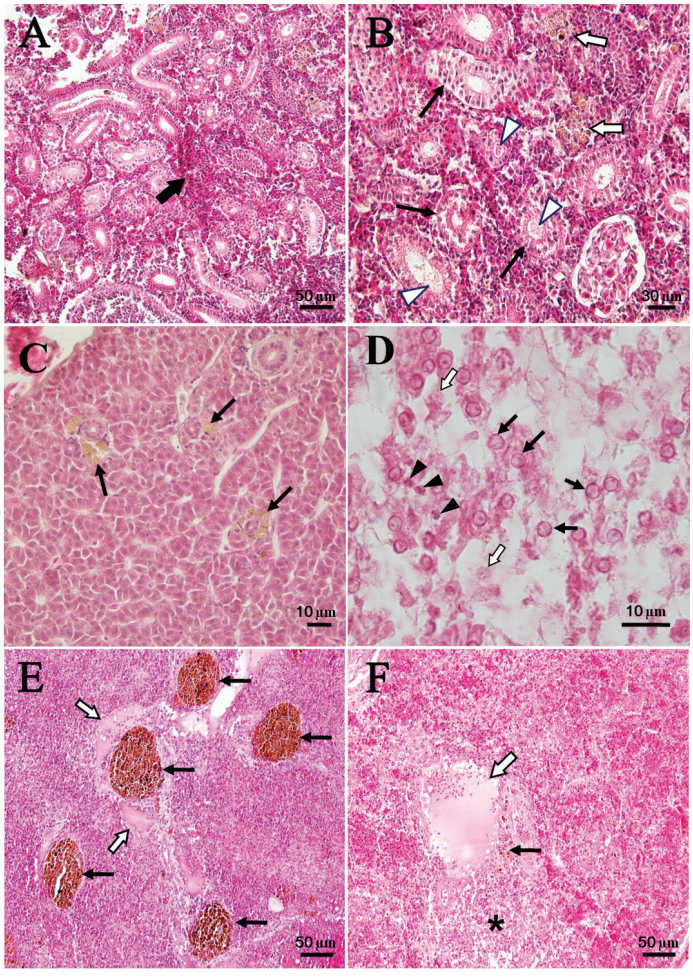

Figure 3: Histopathology of diseased mud loach, *Misgurnus mizolepis*, experimentally infected with *Aeromonas sobria*. On day 5 post-infection (PI), the kidney exhibited extensive parenchymal hemorrhage (A, arrow), severe tubular necrosis (B, arrows), accumulation of proteinaceous substances in the tubular lumen (B, white arrowheads) and hemosiderin granules (B, white arrows). On day 3 PI (C), the liver displayed atrophied cells and some hemosiderins (arrows) nearby bile ducts. Hepatocytes showed severely necrotic foci with karyopyknosis (arrowheads), karyolysis (white arrows), and hyperchromatosis (arrows) of the nuclear membrane at day 5 PI (D). Splenic pulps presented large hemosiderins (arrows) surrounded by fibrins with some glassy eosinophilic materials (white arrows) at day 3 PI (E). On day 5 PI, the spleen (F) showed severe hemorrhage, eosinophilic materials (white arrows), deposition of hemosiderins (arrows), and destruction of sheathed tissue (asterisk)

Different superscript letters indicate significant differences among experimental groups. Single and double asterisks indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively, between experimental and control groups.

TLC, Total leukocyte count; TP, total protein; ALP, alkaline phosphatase; Ca, calcium; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase

Leukograms showed the predominance of lymphocytes and neutrophils during the experimental period (Figure 2). On day 1 PI, neutrophil and monocyte numbers in infected fish increased in relation to control whereas there were no significant changes in the number of lymphocytes and eosinophils. On day 3 PI, there were significantly more lymphocytes and monocytes in infected fish than in control fish, while there were significantly less neutrophils. On day 5 PI, lymphocytes continuously increased whereas monocytes decreased. No significant changes were observed in the number of eosinophils between infected and control groups.

As evidenced in Table 2, at the beginning of the experiment, 39% of the circulating erythrocytes ranged from 7.6 to 8.0 μm, but by day 5 PI, the size of the erythrocytes of the infected group had decreased to 13%, compared to the size of the erythrocytes of the control group. These erythrocytes appeared as elliptical cells with an oval, central or nearly central, dark purple-stained nucleus and a blue-gray stained cytoplasm (data not shown). At day 3 PI, 4% of the measured erythrocytes ranged from 6.1 to 6.5 μm, but on day 5 PI erythrocytes within this range increased to 18% of the total number of erythrocytes. These cells were round with centrally positioned, round nuclei in a dark-blue-stained cytoplasm; they were considered reticulocytes based on Gao *et al.* (14) classification.

Histopathology

No histopathological symptoms were observed in tissues of infected fish at day 1 PI. However, on day 3 PI, there was extensive hemorrhaging in the kidney with generalized necrosis of hematopoietic tissue and accumulation of eosinophilic proteinaceous substances in the lumen of necrotized renal tubules (Figure 3A and 3B). Extensive hemorrhage in the parenchyma and peritubular region with renal tubular necrosis continued until day 5 PI. Atrophy of hepatic cells and some hemosiderins appeared nearby bile ducts at day 3 PI (Figure 3C). On day 5 PI, liver cells showed severely necrotic foci with karyopyknosis, karyorrhexis, karyolysis and hyperchromatosis of the nuclear membrane (Figure 3D). In the spleen, pulps were hemorrhaged, sheathed tissue was destroyed, and many hemosiderins were surrounded by fibrins with some glassy eosinophilic materials (hyaline droplet degeneration) (Figure 3E and 3F) on days 3 to 5 PI. Control fish showed no histological lesions in visceral organs (data not shown).

Discussion

Bacterial pathogens such as *Aeromonas* spp. cause significant hematological and histological changes, which lead to mass mortalities of freshwater fish (7, 15, 16). In the present study, we found hematological and histological alterations in mud loach infected with *A. sobria*.

Erythrocytes contain hemoglobin, which facilitates the transportation of oxygen by reversibly bonding the respiratory gas, thereby increasing its solubility in blood (17). Both Ht and Hb are good indicators of fish oxygen transportation capacity allowing establishing a relationship between the oxygen available in the environment and fish health (18). Scott and Rogers (19) reported that reduced Ht and Hb levels are due to the mobilization of erythrocytes in combination with poor hemoglobin from the spleen to other hematopoietic organs, resulting in hypochromic anemia. Infections by *Aeromonas* spp. damage internal organs, especially hematopoietic organs such as the spleen and kidney (16, 20, 21). Baruah *et al.* (22) suggested that anemia might be due to hemodilution caused by a loss of body fluid from hemorrhagic or necrotic lesions. In the present study, Ht and Hb levels were considerably reduced in *A. sobria*-infected fish compared to healthy fish, and this was also observed in rainbow trout, *Oncorhynchus mykiss* (15), common carp, *Cyprinus carpio* (23), striped snakeheads, *Channa striatus* (24), and Nile tilapia, *Oreochromis niliticus* (25) infected with different bacterial pathogens. In the present study, mud loach infected with *A. sobria* showed severe hemorrhage with necrosis and deposition of hemosiderins (hemolysis) in the kidney and spleen. Overall, these results indicate

that reduction of Ht and Hb levels observed in infected fish might be attributed to the impairment of oxygen carrying capacity by *A. sobria* and/or to damage in hematopoietic organs.

Infected mud loach showed an increase in the proportion of reticulocytes (immature erythrocytes) on day 3 PI, which continued to increase until day 5 PI. The appearance of immature erythrocytes in the circulation might reflect the pathophysiological alterations that occur as infected fish attempt to maintain homeostasis (26).

Leukocytes are important components of the immune system and play critical roles in the defense against pathogen invasion (13, 17, 27). In fact, the increase in TLC during bacterial infection is correlated to the stimulation of defense functions against pathogens (13, 17, 28). MacArthur *et al.* (29) reported the migration of leukocytes from the blood and organs into sites of damaged tissue in European plaice, *Pleuronectes platessa* infected with *Vibrio alginolyticus*, corresponding to an acute cellular inflammatory response. In the present study, the TLC in infected fish increased until day 3 PI suggesting that *A. sobria* infection enhanced immunological responses by increasing leukocyte numbers in the early stage of the infection. However, on day 5 PI, there was a decrease in TLC and severe necrosis of the kidney tissue. This TLC reduction in infected mud loach might be attributed to fish losing the ability to produce leukocytes due to damage in hematopoietic tissues, which may compromise immunological functions such as phagocytosis in later stages of the infection.

The different types of leukocytes were affected by *A. sobria* infection. Neutrophils play a key role in the early stages of inflammatory response and are involved in cellular immune response, migrating to injury sites such as those resulting from bacterial or parasitic infections (11, 13, 17, 30). Pathiratne and Rajapakshe (11) and Ranzani-Paiva *et al.* (31) reported that fish affected by ulcerative syndromes and *Mycobacterium marinum*, respectively, showed more neutrophils than healthy fish. In contrast, Nile tilapia infected with *Enterococcus* spp. and striped snakehead infected with *A. hydrophila* exhibited fewer neutrophils than healthy fish (24, 25). In the present study, neutrophils' number increased in infected mud loach on day 1 PI but was significantly lower than that of control fish on day 3 PI. Thus, the increase in neutrophils in the early stage of the infection might be associated with the defense mechanism used to initiate and potentiate responses against the invading pathogen; as neutrophils migrate from the blood to damaged tissues to remove pathogens and cellular debris while infection develops, there is a decrease in the number of neutrophils circulating in blood.

Monocytes are phagocytic cells derived from macrophage migrating from the blood to various tissues (25, 30). In the present study, an increase in monocytes' percentage was observed in infected fish on day 1 PI and it remained high until day 3 PI. This increase in monocytes might be caused by their migration from hematopoietic tissues into circulating blood to orchestrate immune responses during the early stage of infection. At the end of the experiment, the number of monocytes in infected fish was similar to that before the infection. This reduction in the monocyte population at the later stage of the infection might have resulted from their accelerated movement from the blood to damaged tissues in order to remove cell fragments and pathogens.

Lymphocytes are usually the most common type of leukocytes present in fish blood (32). In Korean catfish, *Silurus asotus* infected with *Edwardsiella tarda*, a high number of lymphocytes were observed (13). Similarly, we observed that the number of lymphocytes in *A. sobria*-infected mud loach considerably increased in the later stage of the infection. Although the mechanism underlying the increase in lymphocyte number in mud loach infected with *A. sobria* is still unclear these cells seem to work as immune-competent cells, as suggested by Martins *et al.* (25).

Eosinophils possess the necessary cellular machinery (innate immune receptors, pro-inflammatory cytokines, antibacterial proteins, and DNA traps) for an efficient antibacterial response (33). Yazdanbakhsh *et al.* (34) reported that eosinophils can phagocytize and kill several bacteria including *Staphylococcus aureus* and *Escherichia coli*, although not as efficiently as neutrophils. Striped snakehead fish affected by epizootic ulcerative syndrome showed increased eosinophils percentage (35). However, in the present study, no difference was found in eosinophil numbers between infected and control fish. Thus, eosinophils seem to play a very limited role in bacterial responses, especially in systemic bacterial acute infections.

The reduction of TP in fish reflects the impairment

of protein re-absorption in renal tubules (36), and Řehulka and Minařík (37) suggested that protein loss in brook trout, *Salvelinus fontinalis*, affected by columnaris disease could be caused by external lesions, kidney damage, and reduction of protein synthesis in the liver. We observed that the renal tubules of infected mud loach were severely necrotized and presented abundant hemorrhage, suggesting that TP reduction might be due, at least partially, to kidney damage and protein loss after *A. sobria* infection. However, other mechanisms such as reduced hepatic protein synthesis and renal re-absorption should be considered.

As a leakage enzyme, AST highest concentrations are found in the cytoplasm of hepatocytes of all species, in the cytoplasm with only about 20% located within mitochondria (38, 39); ALT is present in high concentrations in liver and to a lesser extent in the skeletal muscle, kidney, and heart (40). The levels of these enzymes increase in the plasma when hepatic cells are damaged or their membranes disrupted, allowing the enzymes to leak out (22, 41). Řehulka and Minařík (37) assumed that increased ALT levels signaled liver cell insufficiency, that and increased AST levels signaled a serious damage to the liver with the release of the mitochondrial AST in brook trout affected by columnaris disease. A significant increase in both enzymes has also been reported in various fish infected with *Serratia liquefaciens* (41), *Aphanomyces invadans* (22), Cyprinid herpesvirus-2 (42), and *A. salmonicida* (43). According to Jeney *et al.* (44), increased AST levels in the plasma reflected tissue damage, probably due to the necrosis of hematopoietic tissues in Wels catfish, *Silurus glanis*, infected with a new serotype of *Rhabdovirus carpio*. Because ALP is an induced enzyme that is attached to cell membranes and synthesized by many tissues such as liver, pancreas, kidney, and intestine (38, 39), it plays an important role in the transport of metabolites across membranes and is involved in the synthesis of several enzymes (45). Columnaris disease (37) and *V. anguillarum* (46) or *E. tarda* (13) infections cause a decrease in ALP levels that correlates with signs of anemia. Increased AST and ALT levels and a reduced ALP level in the case of vibriosis have been described in Coho salmon, *Oncorhynchus kisutch* (46) and Atlantic salmon, *Salmo salar* (47). In addition, Řehulka and Minařík (37) observed an increase in AST and ALT and a decrease in ALP activities in brook trout affected by columnaris disease. Similarly, *A. sobria*-infected mud loach showed an increase in AST and ALT, and a decrease in ALP levels. Histologically, *A. sobria* caused severe damage to liver and kidney tissues, suggesting that changes in AST, ALT, and ALP levels are directly related to such damages (cell membrane damage) in internal organs. Hence, *A. sobria* seems to induce changes in AST, ALT, and ALP activities in the blood, as observed for other microbial infections.

Calcium (Ca) is known to combine with proteins, mainly with albumin and 30-40% of the total plasma calcium in freshwater fish is bound to proteins (48). Thus, changes in plasma proteins affect total Ca concentration, and reduced. Ca levels are related with hypoproteinemia, particularly hypoalbuminemia (37). In the present study, TP and Ca levels were reduced in infected mud loach, similar to that reported for brook trout infected with *Flavobacterium columnare*. Our results suggest that *A. sobria* infection causes hypocalcemia by decreasing the levels of protein-bound Ca.

Urea discharge through the kidney and the increase in its level are associated with an increased protein catabolism, due to fasting, infection and blood loss (37). Řehulka (15) demonstrated that rainbow trout infected with *Aeromonas* spp. had an elevated urea level, and proposed that the high urea value in infected fish arose from hypovolemia due to the decrease in glomerular filteration and increase of blood urea nitrogen absorption. An increase in UA level was also recorded in brook trout with columnaris disease (37). The UA level in the plasma of infected mud loach was significantly increased compared to that of control fish, indicating that kidneys of infected fish were damaged, which was consistent with the histopathological observations. However, Philip and Rajasree (49) suggested that the increase in UA values observed in common carp exposed to cypermethrin was caused by the increase in amino transferase activity with a concurrent increase in deamination capacity of some proteins.

Lactate dehydrogenase is found in the cytoplasm of most cells and it is released into the extracellular space and blood when tissue cells are injured (38). An increase in LDH has been reported in salmonids infected with pathogenic bacteria (13, 37). In the present study, we found that *A. sobria* caused a significant increase in LDH levels and severe necrosis of liver and

kidney tissues in mud loach, suggesting that the histopathological features found in liver and kidney might be associated with an elevation in the catalytic concentration of LDH, as proposed by Řehulka and Minařík (37).

The level of GLU in the plasma of infected mud loach was reduced compared to that of control fish. Aydin *et al.* (8) and Yu *et al.* (13) proposed that the decreased GLU levels found in rainbow trout infected with *Campylobactor cryaerophila* and Korean catfish infected with *E. tarda*, respectively, was linked to hypoglycemia, due to the decrease in the activity of liver glicogenolytic enzymes under the stress caused by the infections. Columnaris disease and *S. liquefaciens* infection also caused a reduction in the GLU values of brook trout (37) and rainbow trout (41). The reduction in the GLU level, along with the reduced activity of AST, suggested decline of hepatic protein synthesis during infection stress. In the present study, we found that *A. sobria* caused severe histological damage to liver tissue, indicating that the reduction of GLU level in plasma of infected mud loach might account for the degeneration and necrosis of hepatic tissues that lose their glycogen storage ability and metabolic functions.

In summary, we found that *A. sobria* infection causes hematological and biochemical changes such as anemia, hypoglycemia, hypoproteinemia, hypocalcemia, increase in AST, ALT and LDH activity, increased concentrations of urea and ALP reduction. These alterations lead to detrimental consequences such as compromised disease resistance, tissue damages and low survival rates. Hemato-biochemical changes were also partially accompanied by histopathological changes. Although it is difficult to conclude that natural *A. sobria* infection can cause hematological and biochemical alterations identical to those found under the laboratory conditions tested here, the results of the present study provide a basis for further comparative studies aiming to obtain detailed information on the pathological processes occurring in the blood and visceral organs of mud loach infected with *A. sobria*

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HEMATOLOŠKO-BIOKEMIČNE IN HISTOPATOLOŠKE SPREMEMBE PRI ČINKLJAH (*Misgurnus mizolepis*), EKSPERIMENTALNO OKUŽENIH Z *Aeromonas sobria*

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Povzetek: V raziskavi so bile preučevane hematološko-biokemične in histopatološke spremembe pri činkljah (*Misgurnus mizolepis*), okuženih z *Aeromonas sobria*. Ribe, okužene z 1×10^6 kolonij/ml A. sobria (25 rib x 3 ponovitve v 3 bazenih) in neokužene (kontrolne) ribe (25 rib x 3 ponovitve v 3 bazenih) so bile nastanjene v laboratorijskih akvarijih z ustreznimi parametri vode in bile evtanazirane 1., 3. in 5. dan po okužbi (PI). V petdnevnem poskusnem obdobju so okužene ribe postale letargične, škržne poklopce so hitro odpirale in zapirale, njihove škrge so postale blede in opazne so bile podkožne petehialne krvavitve. Hematološko-biokemični parametri, in sicer hematokrit, hemoglobin, celokupna količina beljakovin, celokupni kalcij, aktivnost alkalne fosfataze (ALP) in nivo glukoze so bili znatno zmanjšani, medtem ko so bili sečnina (UA), aspartat aminotransferaza (AST), alanin-aminotransferaza (ALT) in laktat dehidrogenaza (LDH) v okuženih ribah opazno povečani v primerjavi s kontrolno skupino rib. Skupno število levkocitov je bilo večje pri okuženih kot pri kontrolnih ribah po 3. dnevu PI, vendar se je 5. dan PI dramatično zmanjšalo. V beli krvni sliki so med obdobjem poskusa prevladovali limfociti in nevtrofilci. Histološka slika ledvic in vranice je pokazala obsežne krvavitve s celično nekrozo. V hepatocitih so bila vidna močno nekrotična žarišča s kariopiknozo, karioheksijo, kariolizo in hiperhromatozo jedrne membrane. Okužba z *A. sobria* je povzročila hematološko-biokemične spremembe, kot so anemija, hipoglikemija, hipoproteinemija, hipokalcemija, povečane aktivnosti AST, ALT in LDH, povečane koncentracije UA in zmanjšana aktivnost ALP, kar je privedlo do zmanjšane odpornosti na bolezen, poškodbe tkiva in nizke stopnje preživetja.

Ključne besede: *Aeromonas sobria*; hematologija; histopatologija; *Misgurnus mizolepis*

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Original Research Article

OCCURRENCE AND TRANSPLACENTAL TRANSMISSION OF *Anaplasma marginale* IN DAIRY CATTLE

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Abstract: Bovine anaplasmosis, caused by *Anaplasma marginale*, is a non-contagious tick borne disease. The main objective of the current study was to investigate comparative frequency of anaplasmosis in three different cattle breeds (European breed/ Holstein Friesian and Jersey breed, indigenous breed and cross breed (1st and 2nd pedigree) and transplacental transmission using real time polymerase chain reaction for detection. Of the total 96 blood samples analyzed, our results indicated an overall incidence 45.83% (44/96) of *A. marginale* with highest incidence 62.5% (20/32) in European breed, followed by 42.4% (14/33) in cross breed and 35.4% (11/31) in indigenous breed. Most importantly, our results indicated that 13.7% (4/29) calves were found positive for the presence of *A. marginale* indicating transplacental transmission. Furthermore, indirect ELISA revealed an overall incidence rate of 34.3% (33/96) more likely indicating current or previous exposure. Finally, Giemsa staining determined that 15% (15/96) animals were found positive by examining red blood cells. Statistical analysis showed significantly higher ($P<0.05$) incidence of European breed as compared to crossbreed and indigenous breed of cattle, while non-significant ($P>0.05$) difference was found among the crossbred and indigenous breed of cattle. Moreover, non significant ($P> 0.05$) effect of age group was observed on the incidence of *A. marginale*. White blood cell count and mean corpuscular volume were significantly ($P<0.05$) higher in infected cattle, while, red blood cells, packed cell volume hemoglobin concentration, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration (MCHC) were significantly ($P<0.05$) higher in non-infected as compare to infected animals.

Key words: *Anaplasma marginale*; cattle breeds; enzyme linked immuno sorbent assay; real-time polymerase chain reaction; transplacental transmission

Introduction

Anaplasmosis is caused by *Anaplasma marginale,* an intra-erythrocytic rickettsial organism (1, 2). Bovine anaplasmosis alone, or in combination with babesiosis, is responsible for major economic losses to cattle farming due to adverse affects on the performance of cattle (3). Though exact estimate of economic losses due to anaplasmosis in the study area have not been documented but an estimated annual loss due to anaplasmosis in the US alone amounts to $100 million and includes 50000 to 100000 cattle deaths (4). In fact, anaplasmosis causes weight and production loss, delays growth, lowers fertility in bulls, abortions in females and may consequently lead to death (5, 6). It has been projected that tick borne diseases cause US $ 13.9- 18.7 billion losses annually and about 80% population of cattle is at danger of tick borne diseases throughout the world (7).

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Transplacental transmission of anaplasmosis is well documented in addition to its transmission by tick saliva after replication within the tick gut or mechanically through biting flies and contaminated fomites (8, 9). *Anaplasma marginale* may take 7-60 days for its incubation. Around 10- 90% of the red blood cells possibly will be parasitized in the severe condition of the diseases depending upon the Anaplasma strains and exposure of the host. Generally, all ages of cattle can be infected by *Anaplasma marginale,* but the intensity of the disease is age dependent. It has been previously observed that generally young calves were found less susceptible to clinical infection, but those between 1 and 2 years of age suffer from acute form and often become fatal with mortality range of 29 to 49% (10). Fluctuated intensity of red blood infected circulating cells has been observed between the 10-14-days of infection periods (11). *Anaplasma marginale* infected cattle will remain carrier and will transmit infection to susceptible cattle throughout their entire life (12).

Both direct and indirect methods are used for the diagnosis of anaplasmosis, whereas, direct microscopy is used for routine screening, however, DNA based detection through polymerase chain reaction (PCR) is highly sensitive.

Indirect enzyme-linked immunosorbent assay (ELISA) is a widely used technique for detecting and quantifying antibodies in serum samples, especially during epidemiological studies showing a sensitivity of approximately 96.9% (13). In Pakistan, *A. marginale* has been reported from various parts of Punjab (14, 15) and Sind province (16), but the issue has been under reported from parts of Khyber Pakhtunkhwa Pakistan. Furthermore, there has been increasing reports of susceptibility of European breeds to anaplasmosis infection. Moreover, current trends of ticks control have been mainly focused on to restrict transmission through vectors, while no attention is given to the parental screening for the carrier status. The current project was thus designed with an aim to evaluate the overall incidence of bovine anaplasmosis using sensitive detection tool such as real time PCR, to compare the susceptibility levels of European breed and indigenous breeds and to find the extent of transplacental transmission.

Materials and methods

Selection of animals

The current study was carried out during September 2014 and May 2015. Blood samples were collected from cattle breeds that were either indigenous (n=31), European breed (Jersey and Friesian; n=32) and cross-bred (indigenous cross European; n=33) of age groups 0-12 month, 13 months - 3years and over 3 years, respectively. Out of the total 96 animals, 67 were non pregnant and 29 were pregnant (last trimester). The pregnant animals were followed until parturition and calf was sampled at day 1.

Ethics and animals rights

The study was approved by the ethical committee of the university, The University of Agriculture, Peshawar Pakistan and all procedures were essentially carried out according to ethical rights laid down in 1964 Declaration of Helsinki and its later amendments.

Collection of blood samples and hematology

Fresh blood from both ear and caudal vein of each animal was collected. A total of 10 ml of blood from jugular vein of each animal was collected to isolate serum (in non EDTA containing vacutainer tubes); hematology plus PCR (in EDTA containing vacutainer tubes). Blood was collected from pregnant cows during last trimester and from new born calves on day 1st of birth. Collected samples were kept dry and were processed in the Laboratory of Parasitology at the department of Animal Health as well at Veterinary Research Institute (VRI), Peshawar for microscopic examination and PCR analysis. Serum was separated by centrifugation at 3000 rpm for 10 min after incubation at room temperature, and isolated serum was stored at 4-8°C.The EDTA containing blood was also stored at 4°C and shifted to VRI, Peshawar within two hours for hematology and real time PCR analysis. The hematology was performed through hematology analyzer URIT-2900 VET PLUS (China).

Preparation of blood Smear

Two thin blood smears were made from each blood sample and stained with Giemsa stain after drying for microscopic confirmation of *A. marginale* as described earlier (17) and was examined under the microscope. Samples were regarded positive based on finding of the dark blue dot shaped bodies (organism) in the margins of red blood cells using 100x oil emersion lens (18).

Serological detection of A. marginale

An indirect ELISA kit (SVANOVIR® *A. marginale* –Ab, Uppsala, Sweden) was used to detect antibodies against *A. marginale* in collected blood-serum according to the manufacturer's instruction.

Real Time PCR for Anaplasma marginale detection in cattle

Chromosomal DNA of *A. marginale* was isolated using DNA isolation kit DN easy® blood and tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. Real time PCR was performed as described earlier (18) with slight modifications. In this PCR the major surface protein (msp1α) gene, a stable and ubiquitous gene marker specific to *A. marginale*, was targeted using primers combination: Forward primer TTGGCAAGGCAGCAGCTT and Reverse primer: TTCCGCGAGCATGTGCAT. The template DNA samples (1-5µg/µL) were mixed with 15 µl of *Sso*fast super mix and 2.5 µL of 10 pmol of each Primer. In addition, positive control (15 µL of *Sso*fast super mix (SsoFast TM®-Super Mix-BioRad, USA) + 5 µL of DNA + 2.5 µL of 10 pmol of each Primer) and negative control (15 µl of Sso fast super mix + 5 µL of PCR water + 2.5 µL of 10 pmol of each primer) were also added for the PCR reaction in CFX96 thermal cycler (BioRad, Hercules, CA, USA). The real time PCR conditions were set to (i) Initial denaturation at 95 ^{0}C for 1min (ii) cyclic denaturation at 95 ^{0}C for 1 min (iii) annealing at 60 ^{0}C for 1min (iv) extension at 72^{0}C for 1 min and (v) final extension at 72 ^{0}C for 5 min. Primers were synthesized by Invitrogen.

Statistical Analysis

Data was nalyzed using SPSS version 16.0 to determine significant differences of *A. marginale* occurrence among breed and age groups of both pregnant and non-pregnant cattle. Logistic regression was used specifically for knowing the frequency of *A.marginale* incidence. Means of various blood parameters were compared through ANOVA using General Linear Model and were ranked using Duncan's Multiple Range Test.

Results

Incidence of A. marginale in different cattle breeds

To determine the overall incidence of *A. marginale,* we performed real time PCR targeting gene msp1α as described earlier (18). Our results indicated that 44/96 (45.83%) samples were found positive for *A. marginale* indicating a high prevalence (Table 1). We further tested these samples with two other commonly used diagnostic techniques such as indirect ELISA and direct examination of blood smear with Giemsa stain to confirm exposure and sub/clinical form to observe the presence of parasite in the blood smear. Our results indicated that using ELISA, an overall 33/96 (34.3%) samples were identified positive, while only 15/96 (15.63%) could be declared positive using direct thin smear microscopy approach (19) (data not shown) indicating that these animals were sick of anaplasmosis. Secondly, this also indicates, that real time PCR is more sensitive than the traditional ELISA and blood smear techniques. Overall, our results indicated high prevalence (45.83%) of bovine anaplasmosis in cattle and high sensitivity, as expected, of real time polymerase chain reaction (RT-PCR) detection as compared to ELISA and direct microscopy.

Breed wise susceptibility

Our results indicated that, based on the RT-PCR, 20/32 (62.5%) European breeds, 14/33 (42.4%) of cross breeds and 11/31 (35.4%) indigenous breed were found infected with *A. marginale* (Table 1). Importantly, European breeds were living in a separate shed although within the same premises of the cross breeds. However, most of the indigenous breeds that were sampled were of small herd and routinely kept by the local farmers. Statistical analysis showed significantly

higher (P<0.05) incidence of *A. marginale* in European breed as compare to crossbred and indigenous breed of cattle, while non-significant (P>0.05) differences were found among the crossbred and indigenous breed of cattle (Table 1 and 2). Furthermore, statistical analysis showed no significant (P > 0.05) effect of breed age group on *A. marginale* incidence (Table 2).

Age wise susceptibility

Interestingly, our results indicated that age group 1 (0-12 months) of European breeds (Frisian and Jersey) were 70% infected, followed by age group 2 (13-36 months) and 3 (>36 months), respectively. However, in cross breed animals, 54.5% of the age group 2, 41.6% of age group 3 and 30% of age group1 was infected. Finally, of the indigenous breeds, 40% of each group 2 and 3, while 27.2 % of age group 1 was infected (Table 1). Interestingly, statistical analysis indicated that no significant (P > 0.05) effect of age group was observed on the incidence of *A. marginale* in the age groups of various breeds of cattle (Table 2).

Above table indicates the incidenc of *A. marginale* in cattle by using real-time PCR. Data indicate that exotic breeds were found highly susceptible, followed by cross and local breeds, respectively. Age group 1: 0-12 months, age group 2: 13-36 months, age group 3: over 36 months.

Above table indicates different blood parameters of infected vs non-infected cattle breeds. Mean of the total of 44 positively diagnosed cattle vs all mean of the 52 negatively diagnosed cattle. Different superscripts within the row are significantly different at p-value (0.05).

Effects on Hematological parameters of infected cattle

Hematological parameters indicated significant differences (P<0.05) among the infected and non-infected animals. White blood cell count (WBC) and mean corpuscular volume (MCV) were significantly (P<0.05) higher in infected as compared to non-infected animals. While red blood cells (RBC), packed cell volume (PCV), hemoglobin concentration (HGB), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were significantly (P<0.05) higher in non-infected as compare to infected animals (Table 3).

Transplacental transmission of A. marginale from infected cattle

We examined a total of 29 day-old calf of already positively declared (for *A. marginale*)

Table 1: Incidence of *A. marginale* in different cattle breeds

Breed	Age group	No. of samples examined	No. of Positive samples	Incidence of *A. marginale (%)*
HF/Jr	1	10	7	70
	2	10	5	50
	3	12	8	66.66
	Total	**32**	**20**	**62.5**
Cross	1	10	3	30
	2	11	6	54.54
	3	12	5	41.66
	Total	**33**	**14**	**42.4**
Local	1	11	3	27.27
	2	10	4	40
	3	10	4	40
	Total	**31**	**11**	**35.4**

Above table indicates the incidenc of A. marginale in cattle by using real-time PCR. Data indicate that exotic breeds were found highly susceptible, followed by cross and local breeds, respectively. Age group 1: 0-12 months, age group 2: 13-36 months, age group 3: over 36 months.

Table 2: Frequency distribution of *A. marginale* in different cattle breeds

Age/Breed	Score	D F	P. Value
Breed(1)	4.706	1	0.030
Breed(2)	0 .400	1	0.527
Breed(3)	5.015	2	0.081
Age group(1)	0.466	2	0.792
Age group(2)	0.449	1	0.503
Age group(3)	0.042	1	0.838
Age group * Breed	3.822	4	0.431
Age group(1) by Breed(1)	2.397	1	0.122
Age group(1) by Breed(2)	1.276	1	0.259
Age group(2) by Breed(1)	0.044	1	0.834
Age group(2) by Breed(2)	0.294	1	0.588

Above frequency distribution results are based on the outcome of real time PCR as depicted in Table 1. Breed 1 = Pure exotic; 2 = Crossbred; 3 = Local breed; Age group 1: 0-12 months; Age group 2: 13-36 months; Age group 3: over 36 months.

Table: 3 Hematological parameters of infected and non-infected dairy cows by *A. marginale*

Parameters		Non infected Mean ± SE	Infected Mean ± SE	P- Value
WBC	(10^9/L)	10.79^{b} ± 0.06	11.81^{a} ± 0.13	0.001
RBC	(10^12/L)	8.08^{a} ± 0.13	4.96^{b} ± 0.35	0.001
HGB	(g/dL)	9.93^{a} ± 0.10	7.76^{b} ± 0.28	0.001
PCV	(%)	32.72^{a}O.19	27.12^{b}0.10	0.001
MCV	(f/L)	45.29^{b} ± 0.05	47.86 ^{a}± 0.02	0.002
MCH	(p/g)	15.89^{a}± 0.01	14.24^{b} ± 0.05	0.001
MCHC	(g/dL)	35.30^{a}± 0.06	33.07^{b} ± 0.10	0.001

pregnant cattle by RT-PCR. Our results indicated that 4/29 (13.7%) of calves were found infected with *A. marginale* indicating reasonable level of transplacental transmission.

Discussion

Bovine anaplasmosis caused by *A. marginale* is a major constraint on livestock production and health of cattle due to its devastating economic impact on the production potential of cattle. Incidence cases of *A. marginale* in diverse range of cattle breeds and evidences of transplacental dissemination from Khyber Pakhtunkhwa, province of Pakistan have been rarely reported. In Pakistan including Khyber Pakhtunkhwa, import of European breeds such as Jersey and Holstein Friesian have been enormously increased during the last five years. Although, the exact data of the number of European breeds in Khyber Pakhtunkhwa does not exist, but based on our own personnel perception and raw data collected from organized commercial farms and breed centers indicated a significant number of European breeds in the province. Although, it is not of surprise that these European breeds would be comparatively more prone to infectious diseases and climatic alterations, however, exact data of such information is lacking in the country and particularly in Khyber Pakhtunkhwa although using all preventive measurement like anti-tick spray. The current study thus certainly gave a first brief overview of the comparative susceptibility of European and indigenous /cross breeds towards *A. marginale* and necessitates the need to ensure and provide safe, clean and supportive environment

in order to draw benefit from European breeds at maximum of their potential. In the current report, we screened a total of 96 blood samples for the presence of *A. marginale* in different cattle breeds in order to determine its incidence, comparative susceptibility of breeds and age of cattle breeds and transplacental transmission. We report on the high occurrence of *A. marginale* particularly in the European breeds and further report on the evidence of transplacental transmission.

Detection of *A. marginale* by PCR is considered highly sensitive and accurate that could even detect the carrier stage in contrary to the classical methods such as direct microscopy and ELISA. Although, there are less expensive alternatives, such as serological tests (ELISA, IFAT) and direct microscopy, but these methods lack specificity and sensitivity as compared to PCR for the detection of *A. marginale*. Moreover, these methods are not able to reveal the exact profile of prevalence of infection at a particular point, as detectable levels of antibodies may remain in the animal for long periods, even after elimination of the infectious agent. As indicated by our current studies a low level 35.41% of detection of *A. marginale* by ELISA and 15.62% detection limit by direct microscopy was observed, corroborated by previous findings (20, 21). Molecular techniques such as PCR, on the other hands due to its higher sensitivity and specificity, indicated higher prevalence of 45% in the current study and 56.9% in Puntarenas Province, Costa Rica (22). Similarly, a prevalence of 48.75% was observed earlier (23).

A. marginale was found significantly ($P<0.05$) higher in the European breeds in comparison to crossbreeds and indigenous breeds of cattle. While non-significant ($p>0.05$) differences were found among the indigenous and crossbreeds of cattle. Similar study was conducted by Marufu et al. (24) who revealed significantly higher incidence of anaplasmosis in European breed (pure Frisians) as compared to the cross and indigenous breeds of cattle. Our findings are also supported by Alim et al. (25), who revealed lower prevalence of anaplasmosis in indigenous cattle as compare to Holstein Frisian cattle, however, there was no significant difference of *A. marginale* prevalence among different age groups of cattle as described earlier. Our findings are also supported by Hamou et al. (26) that described no significance difference of *A. marginale* among difference age group of cattle. It is known that breed improvement for high production of milk and meat may compromise the immune system of the genetically improved animals (27) most likely making them vulnerable to infectious diseases.

WBC and MCV were significantly ($P<0.05$) higher in infected as compared to non-infected animals. However, RBC, HGB, PCV, MCH and MCHC were significantly ($P<0.05$) lower in infected as compared to non-infected cattles. The lower number of RBCs observed is more likely due to the activated immune system against the parasitized cells to eliminate them from the body. Since, destruction of RBCs occur more rapidly due to phagocytosis of the infected RBCs and demand of the body increase for RBC resulting selective pressure to release immature RBCs from bone marrow. Because, the immature RBCs harbor larger size than mature red blood cells so resulting in increased MCV (28).

Transplacental transmission of *A. marginale* has been reported previously with a wide range indicating varying degree of vertical transmission in different animals (29, 30). We indicated a 4% transplacental transmission following those animals that were detected anaplasma positive during their pregnancy. However, in most of the cases, we were not sure of the exact day and time of infection though. Other reports indicated a comparatively higher incidence (12.5 %) in cattle calves, but they did not mention the status of the diseases in the mother and the time of the infection when the mother was infected. The feature of uterine transfer has implication in the epidemiology of anaplasmosis in infection free areas. Interestingly, we observed a comparatively lower prevalence of transplacental transmission of *A. marginale* in infected pregnant cattle to their calves as compared to previous reports of 10.5%-12.5% (31,32) Similarly, in South Africa, a transplacental transmission was found to be 15.6% among calves borne from cows showing chronic infection or primo-infected during pregnancy (33), however, from clinically infected animals while showing clinical signs, transplacental transmission was noted considerably as high as 86.4% (32/37) in calves (34). The mechanism of transplacental transmission needs further investigation to understand its dissemination. The higher transplacental transmission rate may be due to the clinical or acute infection of anaplasmosis.

European breeds (Holstein Friesian and Jersey) were found more susceptible to *A. marginale* as

compared to the cross (1st and 2nd pedigree) and indigenous breeds. Using RT-PCR indicated a higher 45.83% (44/96) prevalence of *A. marginale* with highest incidence of 62.5% (20/32) in European breeds, followed by 42.4% (14/33) in cross breeds and 35.4% (11/31) in indigenous breeds. ELISA indicated that 35.4% had exposure to the parasite, while 13.7% were found positive for parasite in their blood samples. Finally, detection of *A. marginale* in day-old calves (pure exotic breeds) indicated its ability to transmit vertically.

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POJAVNOST IN PRENOS *Anaplasma marginale* PREKO POSTELJICE PRI MLEČNEM GOVEDU

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Povzetek: Goveja anaplazmoza, ki jo povzroča *Anaplasma marginale*, je bolezen, ki ni kužna in jo prenašajo klopi. Glavni namen študije je bil raziskati in primerjati pogostost anaplazmoze pri različnih pasmah goveda in sicer dveh evropskih pasmah (holstein-frizijske pasma in pasma jersey) pri avtohtoni pasmi ter pri križancih med evropskimi pasmami in avtohtonimi pasmami (1. In 2. generacija križancev) ter prenos preko posteljice z uporabo verižne rekacije s polimerazo v realnem času (PCR). Od skupno 96 pregledanih vzorcev krvi so rezultati pokazali skupno incidenco 45,83 % (44/96) *A. marginale* z največjo incidenco 62,5 % (20/32) pri evropskih pasmah, nato 42,4 % (14/33) pri križanih živalih in 35,4 % (11/31) pri avtohtonih pasmah. Rezultati so tudi pokazali pozitivno rekacijo na prisotnost *A. marginale* pri 13,7 % (4/29) telet, kar kaže na prenos anaplazmoze preko posteljice. Metoda posrednega testa ELISA, ki pokaže na trenutno ali predhodno izpostavljenost, je pokazala 34,3-odstotno pojavnost pri vseh živalih skupaj (33/96 živali). Proučitev rdečih krvničk z barvanjem Giemsa je pokazalo 15 % (15/96) pozitivnih živali. Statistična analiza je pokazala statistično značilno razliko v pojavnosti anaplazmoze med evropskima pasmama v primerjavi s križanci in avtohtono pasmo ($p < 0,05$), razlika med križanci in avtohtono pasmo goveda pa ni bila statistično značilna. Poleg tega je bil pri pojavnosti *A. marginale* opazen neznaten (statistično neznačilen) učinek starosti proučevanih živali. Število belih krvnih celic in povprečni volumen telesne mase sta bila pri okuženih govedih statistično značilno povečana ($p < 0,05$), medtem ko so bile rdeče krvne celice, koncentracija hemoglobina v celicah in povprečna količina hemoglobina v posameznem eritrocitu (MCHC) v neokuženi skupini višja kot v skupini z okuženimi živalmi ($p < 0,05$).

Ključne besede: *Anaplasma marginale*; pasme goveda; test ELISA; verižna reakcija s polimerazo v realnem času; prenos preko posteljice

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Case Report

HORMONAL INDUCTION OF ABORTION OF A MUMMIFIED FETUS IN A BREEDING WEIMARANER BITCH

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Abstract: The case describes a four-year-old Weimaraner bitch with fetal death resulting in the resorption of two gestation sacks and mummification of a fetus on day 30 of gestation. An ultrasound diagnostic on the 22nd day post-ovulation showed three normal gestation sacks. An ultrasound check-up 3 days later showed only one gestation sack with no heartbeat observed, while the diameter of the gestation sack did not correspond to the gestation period. The uterine wall was intact and the ovaries had *corpus luteum* bilaterally. The clinical status of the patient was physiological. On the 32nd day post-ovulation, an ultrasound showed a big solid cyst on the left ovary. At the bifurcation of the uterus, there was a solid mass (2.56 cm x 3.18 cm) with a lack of fetal fluid. The mass corresponded to mummified fetus. The progesterone level on day 34 of gestation was 31.94 ng/mL. The bitch was negative for *Canine herpes virus*, *Canine bocavirus* and *Brucella canis*. The induction of the abortion started on day 34 post-ovulation with a combination of antiprogestine (Alizine®) and synthetic prostaglandins, (Estrumate®) given subcutaneously and intravaginally administered (Cytotec®). There were certain side effects during therapy, such as tachypnea, polydipsia, nesting and fever. During therapy, the uterine wall, embryo mass, ovaries and eventual occurrence of fluid in the lumen of the uterus were monitored daily. On the 57th day post-ovulation, a mucosal dark discharge occurred. Ultrasound findings showed no presence of fetal mass persistence in the uterus. The bitch showed signs of heat three and half months after the abortion. In the next gestation, it was confirmed by ultrasound that a minimum of five vital fetuses were developing normally. It can be concluded that the described hormonal protocol is the option of choice for breeding bitches in order to avoid unnecessary and undesirable surgery.

Key words: fetal death; bitch; mummification; aglepristone; cloprostenol; misoprostol

Introduction

The case describes a four-year-old female Weimaraner dog which was artificially inseminated (AI) with fresh semen, followed by progesterone (P_4) level evaluation. It was its first insemination and conception. The semen donor was a four-year-old Weimaraner with a good breeding history which had resulted in four litters. During both inseminations (on day one and day three of P_4 level evaluation), the semen was of optimal quality, motility was over 90%, and no signs of spermatozoa abnormalities were observed. Eight days after the first insemination, the herpes vaccine (Eurican Herpes 205®, Merial, Lyon, France) was given s/c (a single dose containing 1 mL of 0.3–1.75 µg of canine herpesvirus antigen).

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Case presentation

The bitch was referred to the Clinic for Obstetrics and Reproduction at the Faculty of Veterinary Medicine in Zagreb, Croatia for

monitoring of the gestation by ultrasound diagnostic. Ultrasonography (US) on the 22nd day post-ovulation (post-OV) showed a minimum of three gestation sacks with a physiological volume of embryonic fluid (the diameter of the gestation sacks corresponded to the gestation period). The internal diameter and length of the gestation sack was measured in the direction of the uterine horn. The maximum length of the embryo size was measured when imaged in the longitudinal plane of the embryo.

Three days later (25th day post-OV), a US check-up showed only one gestation sack but without any heartbeat, and the US measures of the diameter of the gestation sack were not related to the gestation period. The other two gestation sacks were not found. No signs of fluid were observed in the uterine lumen, while the endometrium appeared relatively homogenous and moderately hypoechoic. Both ovaries were of a normal size for metestrus and had several *corpora lutea*. The status of the patient was without any clinical change and without vaginal discharge.

The bitch was on an adequate diet and the owner claimed that the patient had not been exposed to any kind of stress during gestation. The hematological and biochemical blood parameters were within the reference ranges (IDEXX VetAutoread™ Hematology Analyzer, USA; IDEXX, Catalyst Dx Chemistry Analyzer, USA). The P_4 level was 29.06 ng/mL (VIDAS 12®, Biomerieux, France).

On day 32 post-OV, US showed that the right ovary was of a normal size with *corpus luteum*, whereas the left ovary (2.48 cm x 2.32 cm) had a big solid cyst (3.0 cm x 3.13 cm) of irregular shape. In the uterine lumen, at the bifurcation, a solid hypoechoic encapsulated mass (2.56 cm x 3.18 cm) was observed with an absence of embryonic fluid. The intrauterine mass corresponded to the mummified fetus (reduced or without fluid and the absence of fetal heartbeat). The hypoechoic mass appeared as gray-white material without any organized fetal structures within the uterus. Radiography of the abdomen was unremarkable.

Due to the fact that the patient was a breeding bitch, an ovariohysterectomy was not a treatment option. Given that there is a lack of clinical data in literature on the hormonal induction of abortion of a mummified fetus in bitches, we started with the modified treatment protocol for abortion commonly used at the Clinic. The patient was hospitalized at the Clinic in order to observe possible side effects of the treatment and to monitor the uterine lumen by US (Philips, Affiniti 50 G, Philips ultrasound Inc., Bothell, WA, USA). The bitch was always examined in dorsal recumbency. The body of the uterus and both uterine horns were identified at each examination.

During medical management, it is important that the uterine wall stays intact, otherwise surgery (lavage of the uterine lumen) would be the only option. Before starting with the treatment protocol, we measured the P_4 level and performed an intravaginal examination to check the cervix, which was closed without any vaginal excretion. The P_4 level on day 34 of gestation was 31.94 ng/mL.

The bitch was negative for *Canine herpesvirus* (CHV), *Canine bocavirus* (CBoV), and *Brucella canis* based on vaginal smears and blood sample analyses of antibodies against CHV, CBoV and *B. canis*.

The hormonal induction of the abortion started on day 34 post-OV following the application of antiprogestine (a progesterone blocker) and synthetic prostaglandins ($PGF_{2\text{-alpha}}$) respectively, as shown in Table 1.

The antiprogestine aglepristone (Alizine®, Virbac, Carros, France), in doses of 10 mg/kg s/c once per day (QD), was administered over the following two days. US follow-up 24 hours after the second dose of aglepristone showed the same position and same diameter of the mummified fetus. Upon an intravaginal examination, a small amount of colorless mucus discharge was observed.

The day after the application of the second dose of aglepristone, a total dose of 1 mcg/kg (BID - 2 x 0.5 mcg/kg, s/c) of $PGF_{2\text{-alpha}}$ cloprostenol (Estrumate®, Intervet international BV, Zagreb, Croatia) was given. The first application of cloprostenol (day C.1) was applied on day 36 of gestation. Tachypnea occurred 10-15 minutes after cloprostenol application, and eight hours later a small amount of green mucus vaginal discharge was observed. By means of a US examination, it was observed that the fetal mass was 3.13 cm x 2.6 cm in size. The left ovarian cyst was 3.3 cm x 3.0 cm in size, while the left ovary was not visible. Using Color-Doppler US, remarkable perifollicular vascularity was observed. The hematological and biochemical parameters were within the referent ranges.

Table 1: The hormonal treatment of the abortion using combination of antiprogestine and synthetic prostaglandins ($PGF_{2\text{-alpha}}$) described by days with description of side effects.

Gestation day	Day of cloprostenol	Dosage of aglepristone	Dosage of cloprostenol	Dosage of misoprostol	Side effects of hormonal treatment
34	/	10 mg/kg	/	/	/
35	/	10 mg/kg	/	/	/
36	C.1	/	1 mcg/kg	/	tachypnea
37	C.2	/	1.5 mcg/kg	/	tachypnea, minor depression
38	C.3	/	2 mcg/kg	/	tachypnea, depression
39	C.4	/	2.5 mcg/kg	/	tachypnea, depression
40-44	C.5- C.9	/	3 mcg/kg	/	tachypnea, nesting, vocalization
45	C.10	/	3 mcg/kg	2 x 200 mcg	tachypnea, depression
46	C.11	/	3.5 mcg/kg	2 x 200 mcg	tachypnea, depression, fever, vomiting
47	C.12	/	3.5 mcg/kg	2 x 200 mcg	tachypnea, depression, fever, vaginal discharge, inappetence, polydipsia
48-51	C.13- C.16	/	3.5 mcg/kg	2 x 200 mcg	tachypnea, depression, vaginal discharge, inappetence, polydipsia
52	C.17	/	3.5 mcg/kg	1 x 200 mcg	tachypnea, depression, vaginal discharge, inappetence, polydipsia, colic
53-56	C.18-C.21	/	3.5 mcg/kg	/	tachypnea, depression, vaginal discharge, inappetence, polydipsia
57	C.22	/	3.5 mcg/kg	/	tachypnea, depression, dark brown vaginal discharge, inappetence, polydipsia

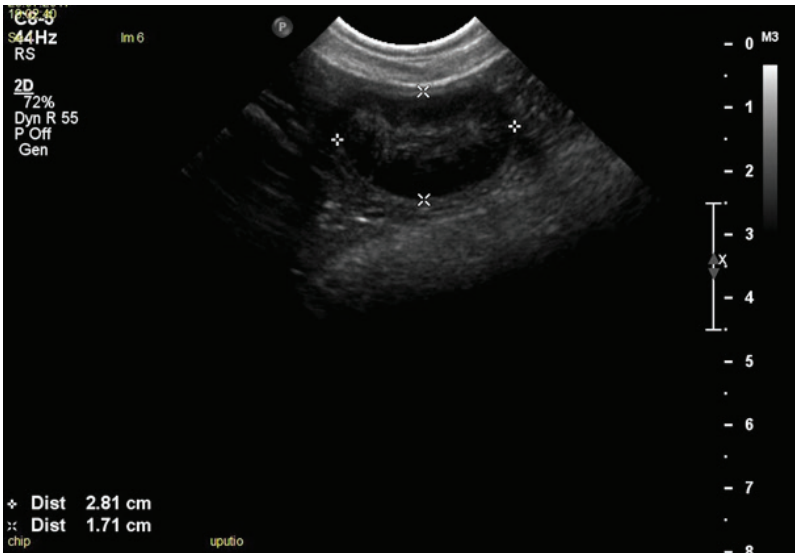

Figure 1: Ultrasound examination on day C.2 shows the intact endometrium without the presence of uterine fluid. The fetal mass decreased (2.81 cm x 1.71 cm) compared to the previous description (C.1) and contained a small amount of anechoic fluid

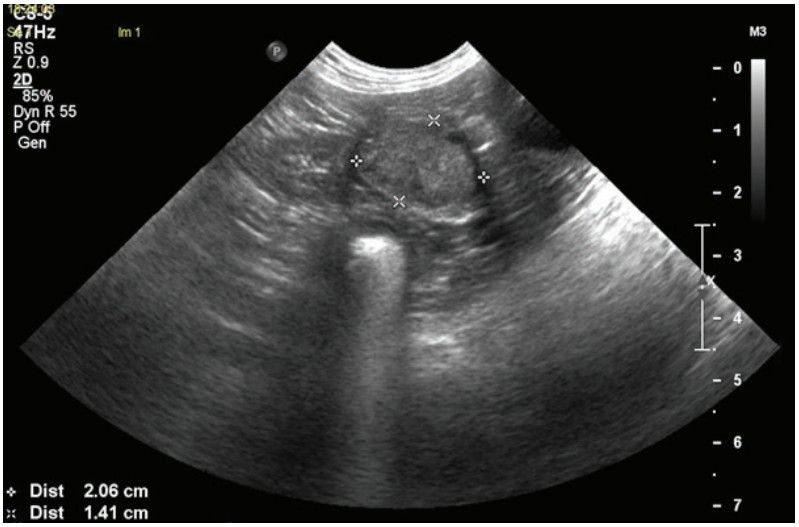

Figure 2: Ultrasound findings on day C.17 show mild endometrial hyperplasia. The fetal mass formation decreased to 2.06 cm x 1.41 cm in size

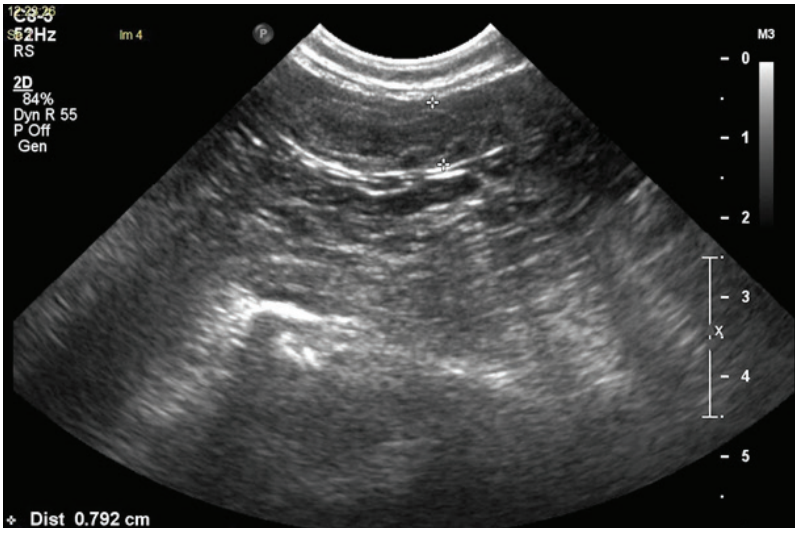

Figure 3: The ultrasound findings performed on day C.22 showed a normal uterus status without fetal formation. The diameter of the uterine corpus is 0.72 cm

On day C.2, the total dose of cloprostenol was increased to 1.5 mcg/kg in two equal doses (2 x 0.75 mcg/kg). A US examination showed an intact endometrium without uterine fluid. The fetal mass had decreased (2.81 cm x 1.71 cm) and contained a small amount of fluid in the lumen (see Fig.1). The patient was clinically stable and showed minor depression. No vaginal discharge was observed.

On day C.3, the total dose of cloprostenol was increased to 2 mcg/kg. Tachypnea occurred again following application. There was no vaginal discharge and the patient's clinical status was unchanged. During vaginal examination, the cervix was still closed.

The total cloprostenol dose on day C.4 was increased to 2.5 mcg/kg. US examination of the fetal mass showed it had decreased (2.4 cm x 1.8 cm). A small amount of colorless vaginal mucus discharge occurred.

On the fifth day (day C.5), a total dose of 3 mcg/kg of cloprostenol was given. Approximately 15 minutes after the first application, the bitch started nesting a few times for a short period. The bitch also started vocalization similar to that during estrus (near the ovulation period).

No vaginal discharge or elevation of body temperature was observed. In addition, the hematological and biochemical parameters were within the referent range.

On day C.6, the same total dose of cloprostenol (3 mcg/kg) was given. By means of US, it was observed that the fetal mass had moved caudally towards the urinary bladder (US check-ups were regularly performed after urination). The shape of the mass became more oval and seemed subjectively larger (2.8 cm x 2.2 cm), and the center area of the mass was observed as anechoic. The uterus was unchanged with no noticeable vaginal discharge. The same dose on days C.7, C.8 and C.9 was applied. US findings were the same as described earlier.

On day C.10, a total dose of 3 mcg/kg s/c of cloprostenol was applied, and a first dose of the synthetic prostaglandin E_1 analog, misoprostol (Cytotec®, Pfizer limited, Kent, United Kingdom; 200 mcg tablets) was administrated intravaginally in order to induce an opening of the cervix. Recommended dose for intravaginal misoprostol for bitches weighing <20 kg bw is 200 mcg while for bitches weighing >20 kg bw is 400 mcg per day as long as vaginal discharge does not occur (1). Since our patient weighed 34 kg bw, we applied misoprostol 2 x 200 mcg per day (BID). The tablet should be ground and melted in a small amount of sterile 0.9% NaCl (0.154 mol/L) in a maximum volume of 2-3 mL, and should be applied intravaginally by AI catheter. During misoprostol therapy, according to our Clinical protocol it is recommended to combine the treatment with systemic PGF_{2alpha} (2). After a second dose of misoprostol, the patient was examined by US and the mass again seemed smaller. No side effects after misoprostol application occurred.

On day C.11, the total dose of cloprostenol was increased (3.5 mcg/kg) and application of misoprostol (2 x 200 mcg intravaginally) was repeated. US examination revealed no change. The bitch became febrile (39.4°C) and vomited twice but still had appetite.

Due to the fact that certain side effects had occurred, on day C.12 the dose of cloprostenol was not increased, and the treatment was the same as the day before. Four hours after the first dose of misoprostol on day C.12, a small amount of dark brownish mucus discharge appeared.

Since US showed no progression, misoprostol therapy was continued. The bitch still had the same body temperature (39.4°C), and depression, inappetence and polydipsia (drinking 4-5 L per day) occurred. Polydipsia could be expected with a cloprostenol dosage above 3 mcg/kg, and the therapy was continued due to the fact that the renal biochemical profile was within the reference range.

Polydipsia and depression continued on day C.13 (3.5 mcg/kg of cloprostenol and 2 x 200 mcg of misoprostol). The body temperature of the patient decreased to a normal range and no vaginal discharge was observed. Depression, inappetence and polydipsia were still present. Checking by means of US, it was noticeable that the mass formation had moved caudally, was positioned in front of the cervix, had decreased in diameter, and was not visible as encapsulated anymore but visible as a fluctuating form.

A small amount of light green mucus discharge occurred in front of the pseudocervix during a vaginal check-up on day C.14 (cloprostenol 3.5 mcg/kg and misoprostol 2 x 200 mcg). The bitch was clinically stable with the same side effects. On days C.15 and C.16, clinical and US findings, as well as the treatment and side effects, were the same as on day C.14.

On day C.17 (cloprostenol 3.5 mcg/kg), the misoprostol dosage was reduced to QD (1 x 200

mcg) due to the fact that on the previous two days the bitch had a small amount of brown vaginal discharge and started presenting colic-like symptoms following misoprostol application. The patient still had tachypnea, depression, inappetence and polydipsia. A US check-up showed endometrial hyperplasia with a small amount of intrauterine fluid proximal to the mass formation (2.06 cm x 1.41 cm) in the corpus (we assumed that it was NaCl saline used for melting misoprostol), as shown in Figure 2. If the aforementioned assumption was correct (presence of intrauterine fluid), it could be concluded that the cervix had opened. Due to the fact that the mass formation had decreased, our hypothesis is that the NaCl saline had passed around the mass formation and filled the uterine corpus.

After the appearance of the fluid in the uterus, on day C.18 cloprostenol (3.5 mcg/kg s/c in total) without misoprostol was administrated. Seven hours later, during a US examination there was no fluid in the uterus and the endometrial wall seemed intact. We assumed that the mentioned change in the endometrial findings could be ascribed to the beneficial PGF_{2alfa} therapy.

A small amount of light brown mucus vaginal discharge appeared from day C.19 until day C.21. On the mentioned days, cloprostenol therapy (2 x 1.75 mcg/kg s/c) without misoprostol administration was continued. US examination revealed the fetal form decreased on a daily basis towards day C.22.

On day C.22, the 57^{th} day post-OV, a huge amount of dark brown vaginal discharge appeared two hours following the first dose of cloprostenol (1.75 mcg/kg s/c).

The US findings showed a normal uterine status without fetal formation (see Fig. 3), which was checked and confirmed by US next day.

The recommendation to the owner was to inseminate the bitch in the next estrus cycle. There was an assumption that the next cycle might be earlier than expected due to the hormonal treatment shortening the luteal phase of the cycle. The bitch showed signs of heat three and a half months (103 days) after the abortion. In the next gestation, on day 35 post-OV, it was confirmed by US that a minimum of five vital fetuses were developing normally, while on the day 55 following conception X-ray findings determined 10 fetal skeletons. On the day 60 post-OV bitch has delivered 10 vital puppies by physiological parturition.

Discussion

Pregnancy is a normal physiological state which can be interrupted by disorders in gestation. Abnormalities in pregnancy are not an uncommon presentation in clinical veterinary practice and may manifest as pregnancy loss or a variety of metabolic conditions that occur during pregnancy (3). Pregnancy loss may occur at any stage of gestation in a bitch (4), and may manifest as embryonic death and resorption, abortion of a live or dead fetus, stillborn pups, or embryo/fetal mummification and retention in the dam's uterus or peritoneal cavity beyond the normal time of parturition (5).

Early fetal death is characterized ultrasonographically by the presentation of fetal anatomy without a heartbeat (3). If fetal death occurs during the first half of pregnancy, resorption or unobserved abortion occurs (6). In our case, the bitch had two types of pregnancy loss. The first type was two embryonic deaths between days 23-25 of gestation, resulting in embryonic resorption, while the second type was an embryonic death with mummification which occurred in the period between days 25 and 32 of gestation.

The resorption of gestation sacks in our case followed the same changes as described in other study (7), such as delayed development of the embryo, delayed detection of embryonic heartbeat, a small embryonic length, and measurement of a slow growth rate by US. However, no studies have reported in detail the features of embryos which subsequently resorbed (7). Some authors (8) mentioned that there are many potential causes of resorption, such as embryonic abnormalities, abnormal maternal environment and infectious agents. There are scientific findings (4) which explain that luteal insufficiency has been hypothesized as a cause of spontaneous abortion in bitches. However, in our case it was not the cause of the pregnancy loss, due to the high P_4 level (31.94 ng/mL).

Some authors (9) have described the causes of resorption of fetal fluid leading to embryo/fetal mummification. The uterus contracts against the fetus, which causes the absorption of fetal fluid and the fetal membranes become shriveled and dried, which happened in our case with the third gestation sack. Based on our US findings of the mummified mass formation, it can be concluded

that embryonic death occurred on day 30 of gestation. Due to the fact that mummification develops in sterile conditions, the future fertility of animals is not affected (10). Scientific study (11) states that fetal mummification is mostly caused by the *Canine herpes virus* (CHV). However, in our case the bitch was negative for CHV.

The type of treatment outcome depends on the following causes: embryonic/fetal death, the stage of pregnancy, and fetal/maternal immunocompetence (4).

The current case is interesting because fetal mummification in female dogs is rare (8), and in most cases the treatment of choice is ovariohysterectomy. One case report (9) describes an unsuccessful attempt to hormonally induce abortion in a bitch with a single dose of estrogen and oxytocin. Since there was no response to such therapy, a cesarean section was performed to remove the mummified fetus while the other report described treatment of the mummification only by cesarean section (10).

Due to the fact that our patient was a breeding bitch and it was her first conception and gestation, we decided to start with a modified hormonally induced abortion of the mummified embryo. However, we were aware that, due to mummification, the uterus response to PGF_{2alpha} treatment would be reduced and medical management challenging.

Conclusion

Given the lack of scientific data regarding hormonal induction of abortion of embryo/fetal mummifications, the current case study has resulted in significant information about a hormonal protocol with a beneficial outcome. In addition, we managed to monitor the bitch by US on a daily basis, which gave us useful information regarding the uterine response to hormonal therapy, appropriate dosages of the hormones applied, the kinetics of decreasing embryo size and formation, and possible side effects of medical management using a combination of aglepristone, cloprostenol and misoprostol.

Finally, it can be concluded that the described hormonal protocol in the current study is the option of choice for breeding bitches in order to avoid unnecessary and undesirable surgery.

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HORMONALNA SPODBUDITEV SPLAVA MUMIFICIRANEGA PLODU PRI PLEMENSKI PSICI PASME WEIMARANEC

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Povzetek: Opisan je primer štiriletne plemenske psice pasme weimaranec, pri kateri je prišlo do smrti več plodov, kar je povzročilo resorpcijo dveh gestacijskih vreč in mumifikacijo ploda 30. dan brejosti. Ultrazvočna diagnostika 22 dni po ovulaciji je pokazala tri običajne gestacijske vrečke. Pregled z ultrazvokom 3 dni kasneje je pokazal samo eno gestacijsko vrečko brez opaznega srčnega utripa, premer gestacijske vrečke pa ni ustrezal času trajanja brejosti. Maternična stena je bila videti nepoškodovana, na obeh jajčnikih pa so bila opazna rumena telesca. Klinični status psice je kazal fiziološke znake brejosti. 32. dan po ovulaciji je ultrazvok pokazal veliko trdno cisto levega jajčnika. Na razcepu maternice je bila trdna masa (2,56 cm x 3,18 cm) s pomanjkanjem fetalne tekočine. Masa je ustrezala mumificiranemu plodu. Raven progesterona v krvi 34. dan brejosti je bila 31,94 ng/ml. Psica je bila negativna na pasji herpesvirus, pasji bokavirus in na Brucello canis. S spodbuditvijo splava smo pričeli 34 dni po ovulaciji s kombinacijo antiprogestina (Alizine®) in sintetičnih prostaglandinov (Estrumate®), ki smo jih dajali podkožno in intravaginalno (Cytotec®). Med zdravljenjem so se pokazali nekateri neželeni učinki, kot so tahipneja, polidipsija, gnezdenje in povišana telesna temperatura. Med zdravljenjem smo dnevno spremljali debelino maternične stene, maso plodu, jajčnike in morebitno pojavljanje tekočine v lumnu maternice. 57. dan po ovulaciji se je pojavil sluzast temen izcedek. Ultrazvočni pregled ni pokazal prisotnosti mase plodu v maternici. Psica je kazala znake gonitve tri mesece in pol po splavu. V naslednji brejosti je bilo z ultrazvokom potrjeno, da se normalno razvija najmanj pet vitalnih plodov. Glede na rezultate lahko predlagamo opisano hormonsko zdravljenje kot možno izbiro za spodbujen splav pri plemenskih psicah, da bi se izognili nepotrebnim in neželenim operacijam.

Ključne besede: smrt plodov; psica; mumifikacija; aglepriston; kloprostenol; misoprostol

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