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**Review Paper**

# PARATUBERCULOSIS (JOHNE´S DISEASE) IN RUMINANTS – AN ONGOING STORY

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**Summary:** Paratuberculosis or JOHNE´s disease is one of the most important diseases in ruminants today. Mycobacterium avium subspecies paratuberculosis (MAP), the cause of paratuberculosis, has a broad host range. Although mainly considered a disease of cattle, sheep and goats, all ruminants, including a large number of wild and exotic species of ruminants, are susceptible. The reported prevalence of infected animals varies by country and region and reaches up to 84.7 % MAP positive dairy herds in some aereas. Although calves are most susceptible to paratuberculosis, older heifers and adult cattle can also become infected. In newborn calves the infection mostly takes place soon after birth by oral ingestion of the organism but MAP can also be found in colostrum and milk of asymptomatic infected cows. Although shedding of MAP is considered not to start before 1.5 years of age, it has been shown, that also young calves can shed MAP and thereby pass infection to other calves in the same environment. The vast majority of herds that acquire MAP do so through purchase of infected animals. The first clinical sign in cattle can usually be seen after calving as a chronic or intermittent diarrhea alternating with periods of normal consistency of faces. In sheep and goats diarrhea is usually absent.

Subclinically infected adult animals do not show visible signs of paratuberculosis, although they carry MAP. The prognosis is poor, the disease leads to emaciation and ends with the death of the affected animal. If clinical symptoms are missing laboratory tests have to be used to confirm or rule out the diagnosis. Many different test systems are available for the detection of MAP, such as Ziehl-Neelsen Staining, faecal culture and Polymerase Chain Reaction (PCR) or the evaluation of antibody levels by Enzyme Linked Immuno Sorbent Essays (ELISA).

Because paratuberculosis is difficult to diagnose and untreatable control and reduction in MAP positive herds and prevention of spreading the disease to negative herds is very important. Hygienic precautionary measures have to be taken to prevent further spreading of the disease in a herd. The mayor aim of this hygienic precaution is to prevent infection of calves and young stock and to purchase MAP- free animals only.

In this article a review about infection, diagnosis and control of this disease is given. New aspects of current researches are combined with basic informations to provide detailed and actual information about this infectious disease for large animal veterinarians.

**Keywords**: cattle diseases; paratuberculosis – diagnosis – epidemiology – preventive and control; *Mycobacterium paratuberculosis* – pathogenicity; cattle; sheep

## History and Etiology

It was in 1895 that Johne and Frotingham (1) first described the disease and demonstrated the presence of acid-fast bacilli in sections of the intestine of infected animals. The presence of these bacilli, indistinguishable from the tubercle bacillus, made them think the condition was an atypical or unusual form of tuberculosis. More than ten years later, in 1906, Bang (2) determined it was not tuberculosis and called it pseudotuberculous enteritis. The disease later became known as paratuberculosis or JOHNE´s disease.

Paratuberculose is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a slow growing, acid fast bacillus dependent on mycobactin containing culture media (3). The

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organism can remain viable for more than 1 year in bovine faeces and black soil, 9 months in pond water and about 160 days in river water. Temperatures of -14°C can be survived for at least one year. Phenolic or cresylic agents have to be used for disinfection. Products labelled as tuberculocidal are also effective against MAP.

## Host range

MAP, the cause of paratuberculosis, has a broad host range. Although mainly considered a disease of cattle, sheep and goats, all ruminants are susceptible. Paratuberculosis has been reported in a large number of wild and exotic ruminants such as deer and mouflon (4), water buffalo (5), and camelids (3). Several reports also describe infections in non-ruminant species like different kind of birds, wild boar (6), mice, rats, hares and fox (7), rabbits (8), bears, different insects and earthworms (9). Pigs and primates (10) are also known to carry MAP, but, as all other monogastriers, do not suffer from clinical signs of paratuberculosis. There are also infrequent reports of infections and increased incidence of specific antibodies against MAP in humans suffering from Morbus CROHN, leading to ongoing discussions about a possible connection between the two diseases (11, 12).

## Occurence

Paratuberculosis has been reported on every continent. Sweden and some states in Australia are the only regions of the world that claimed freedom from the disease over the last years. But recently there were reported findings of paratuberculosis in Sweden although with a very low prevalence (13).

The reported prevalence of infected animals varies by country and region and reaches up to 84.7 % MAP positive dairy herds in parts of Germany (14). 47.0 % positive dairy herds were found by Jakobsen et al. (15) in Denmark, van Leeuwen et al. (16) reported 43.0% positive dairy herds in Canada, and in Wisconsin, USA 50.0 % of the dairy herds were found to be positive for MAP (17). In Austria two representative studies were performed in the years 1994-97 and 2002-03 which showed, that the seroprevalence of antibodies against MAP increased from 6.97 % positive cattle herds in 1994-97 to 19.05 % positive herds in 2002-03 (18).

## Pathogenesis

Most cattle with paratuberculosis are infected as young calves. Approximately 25 % of calves born to cattle with clinical signs of paratuberculosis are already infected in utero. Although calves are most susceptible, older heifers and adult cattle can become infected too (19). The primary site of MAP infection is the ileum, the most distal part of the small intestine. Specialized absorptive mucosal cells (M-cells) overlying small bulges of lymphoid cells near PEYER´s patches ingest MAP and discharge the organism into the subepithelial dome. Macrophages phagocytes the discharged bacillus from the M-cells and migrate into local lymphatics, spreading the infection to regional lymph nodes (10).

## Transmission

The postnatal faecal-oral transmission is the most important means of exposure. After a long incubation period of up to 10 years (10) infected animals shed high quantities of MAP in their faeces. So called “super-shedders” can shed more than 1 million colony forming units (cfu) of MAP per gram of manure (which is more than 20.000 low shedding animals) without showing clinical signs (20). Although shedding of MAP is considered not to start before 1.5 years of age, recent studies showed, that also young calves can shed MAP and thereby pass infection to other calves in the same environment (21, 22). In newborn calves the infection mostly takes place soon after birth by oral ingestion of the organism. The most likely sources are faecal contamination of the calving unit as well as of the udder (if the calf is permitted to suckle). MAP can also be found in colostrum and milk of asymptomatic cows, serving as source of infection. Pasteurizing colostrum and milk is decreasing, but not avoiding this route of infection (19). Transmission via semen, by embryotransfer and from wildlife ruminant reservoirs has also been discussed (8, 23, 24). The vast majority of herds that acquire MAP do so through purchase of infected animals.

## Clinical manifestations

Paratuberculois spreads slowly and it may be years before a herd is recognized as infected. Herds with good management practice may have a lower rate of infection than herds with poor management. Animal losses due to premature death within a herd can be high. Most clinical

cases occur between 3-6 years of age. Young infected cattle do not show clinical signs (silent infection). They seem identical to uninfected herdmates. No examination or tests are available to detect the infection at this stage, but as mentioned before, animals may shed infectious organism into the farm environment.

Subclinically infected adult animals do not show visible signs of paratuberculosis, although they carry MAP. In some cases specific antibodies to MAP and/or positive faecal culture results may be detected. However, most animals subclinically infected with MAP are culled for reasons unrelated to paratuberculosis.

The first clinical sign can usually be seen after calving (10) as a chronic or intermittent diarrhea alternating with periods of normal consistency of faeces. Associated with the diarrhea there is a generalized unthriftiness, a rough hair coat, dry skin and chronic weight loss despite normal or even increased appetite. Most animals test positive on faecal culture and show increased antibody titers at this time.

In advanced clinical disease affected cattle become weak and emaciated. Bottle jaw (intermittent edematous swelling between the rami of the mandible) typifies the disease. Decreased milk production, pipestream diarrhea and cachexia characterize the terminal stage of the disease. In sheep and goats diarrhea is usually absent. The prognosis is poor, the disease ends with the death of the affected animal. Up to now treatment strategies have not been successful. There are no drugs approved for the treatment of paratuberculosis.

## Necropsy findings

In cattle the lesions found at necropsy are characterized by a diffuse granulomatous change, enteritis, without necrosis or reactionary fibrosis. The diseased animal is emaciated, shows serous atrophy of fat deposits, intermandibular edema and serous effusion into body cavitities. Lesions are limited to the gastrointestinal tract and regional lymph nodes. The intestinal mucosa, especially of the ileum, is visibly thickened and shows broad, transverse ("brain-like") folds. This pathognomonic corrugated appearance does not disappear when the intestine is stretched.

## Economic significance

Paratuberculosis has emerged as one of the most prevalent and costly diseases of dairy cattle today. It also affects the beef cattle industry, particularly breeders of purebred cattle. While emacation and death are the final consequences of clinical paratuberculosis, infected cattle also suffer from decreased productivity associated with subclinical disease, infertility, mastitis and increased susceptibility to other diseases. The economic losses include decreased milk production, reduced value at slaughter, increased veterinary treatment costs, costs of disease control programs, as well as loss due to un- or underused production facilities. Indirect costs consists of export testing of live cattle, sheep and goats, testing for interstate movement of animals and funding for paratuberculosis research. Accurate estimates of the overall economic impact of the disease have not been made, because farmers prefer not to acknowledge its presence and enshroud suspect cases with secrecy. Thus it is difficult to ascertain the true prevalence or to estimate financial loss. Whitlock (19) estimated the total loss to exceed $ 1.5 billion annually for the dairy industry in the USA.

## Diagnosis

Clinical cases of paratuberculosis can often be diagnosed based on clinical examination, history and necropsy findings. If clinical symptoms are missing laboratory tests have to be used to confirm or rule out the diagnosis. Unfortunately there exists no test for paratuberculosis with a high sensitivity and specificity, especially in young and subclinical infected animals. Many different test systems are available today for the detection of MAP, such as Ziehl-Neelsen Staining, faecal culture and Polymerase Chain Reaction (PCR) or the evaluation of antibody levels by Enzyme Linked Immuno Sorbent Essays (ELISA). Other methods like Complement Fixation Test, Interferon Gamma Assay, Gel Precipitation Test and Johnin Test which were used in the past are only used for research purposes today.

Microscopic detection of MAP in faeces by Ziehl-Neelsen Stain is a cheap and easy way to confirm diagnosis in clinical cases of paratuberculosis (25) which can be performed in practice. Unfortunately this method has a low sensitivity for the detection of subclinical infected animals (26).

Bacterial Culture from faecal or tissue (intestine, intestinal or liver lymph nodes) samples is one of the most widely used tests for the detection of MAP and serves as a gold standard. Different culture media and decontamination procedures are used to reduce contamination by other

microorganisms and increase culture outcome. Inkubation time for bacterial culture is 8-16 weeks (27) but can be reduced to 23 days by the use of liquid culture systems (28). Specificity of faecal and tissue samples is very high but sensitivity is low, due to the late onset of faecal shedding, intermittend faecal shedding and late occurrence of MAP in regional lymph nodes. To reduce costs, pooled faecal samples from up to 5 cows of the same age can be used in bacterial culture with a reported specificity of 86 % and sensitivity of 96 % compared to individual sampling (29).

PCR can be used to confirm the results of bacteriological culture as wall as to detect the specific insertion sequence IS*900* in faeces, tissue samples, blood and milk. Advantages of PCR are the high specificity of about 90 % and the possibility to gain results in less than 24 hours. Handicaps of the PCR are a low sensitivity of about 53 % (27) and that there is no possibility to differentiate between viable and non-viable MAP cells (27). Several ready-to-use PCR-kits are available today and many research groups and companies are currently working to improve this method.

ELISA for detection of specific antibodies against MAP is probably the most widely used screening test today (27). It is regularly used for blood (serum) samples but can also be used for milk (30). ELISA is easy and quick to perform and automation of the analysis is possible. Reported values for specificity and sensitivity vary between the different test- kits. Collins et al. (31) found a specificity of 84.7 % - 99.8 % and sensitivity of 27.8 – 44.5 % for five different ELISA. One mayor disadvantage of ELISA tests is the late humoral response in animals infected with MAP, leading to a late increase of antibodies and to negative results in young or recently infected animals (10). Nevertheless ELISA is one of the cheapest and most appropriate methods for herd investigations and MAP-control programs. If used for the diagnosis in single animals interpretation of results has to be critical and the test has to be redone, if necessary.

Although diagnostic tools for detection of paratuberculosis have made great progress and current research activities are high, there are still more questions than answers regarding the correct diagnosis, especially in young animals and animals in an early stage of infection. The selection of the right diagnostic tool, the combination of more than one test and reinvestigation of animals and herds are the most important keys to succeed in the diagnosis of paratuberculosis.

## Control and prevention

Paratuberculosis is difficult to diagnose and untreatable. Therefore control and reduction in MAP positive herds and prevention of spreading the disease to negative herds is very important.

MAP positive animals should be culled from the herd as soon as possible and the whole herd has to be retested several times by combination of different diagnostic tests. At the beginning of a test and cull program all animals older than one and a half year should be tested every six month by ELISA and bacterial culture or PCR of faeces. To reduce costs, pooled faecal samples from up to five cows can be used (29). Animals shedding MAP should be removed from the herd immediately, serological positive individuals as soon as possible. For a quick decrease of the infection additional removal of all offspring from positive tested individuals should be performed. As soon as no more MAP shedding animals are found in a herd, test interval can be extended to once a year with the alternative use of ELISA and bacterial culture (PCR).

Additional hygienic precautionary measures have to be taken at positive farms to prevent further spreading of the disease in the herd. The mayor aim of the hygienic precautions is to prevent infection of newborns and young stock and to purchase MAP- free animals only. Optimal hygiene for parturition and immediate separation of neonates from their mothers as well as separation of offspring from antigen and antibody positive and negative mothers are the most important measures to prevent infection of new born animals. Furthermore only colostrum from antigen and antibody free individuals should be fed and only progeny from negative individuals should be used for breeding.

Young livestock has to be separated from adults on pasture for the first two years of the eradication program. To prevent spreading of MAP with feed and water, only plough –land should be fertilised with manure and pollution of water sources with manure has to be avoided. Shaded pastures should not be used for young animals and deer has to be kept away from pastures. The use of separate working equipment for young and sub adult animals and general improvement of cleaning and disinfection should also be performed. Detailed instructions and suggestions are given by Khol et al. (32).

Up to now there do not exist legal restrictions concerning paratuberculosis in livestock trade in Europe. A “European- standstill” for livestock trading and intensive herd testing would help to identify MAP-free regions and to prevent the fur-

ther increase of paratuberculosis in Europe. Furthermore only animals older than 1.5 years, tested negative for paratuberculosis or animals originating from negative tested herds should be introduced to MAP- free areas and herds.

Austria is the first country who will declare clinical paratuberculosis a notifiable disease followed by legally compulsory culling of clinical diseased animals and hygienic directions. This should help to maintain the low MAP prevalence in Austria and to remove MAP shedding animas from the herds (16)

## Conclusion

Paratuberculosis or Johne´s disease is one of the most important diseases in ruminants today. Because of its long incubation period, high economic losses, difficulties in early diagnosis and discussed possible links to Morbus Crohn in humans, paratuberculosis will stay at the top of important diseases for veterinarians in the future. Greatly increased activities are needed to gain more knowledge about the disease and to develop reliable diagnostic tools in young animals.

Austria is the first country which is taking legal actions to prevent further increase of paratuberculosis in livestock but success is only possible if other countries follow and the disease is fought together on a European level.

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## PARATUBERKULOZA (JOHNEJEVA BOLEZEN) PRI PREŽVEKOVALCIH - ZGODBA SE NADALJUJE

W. Baumgartner, J. L. Khol

**Povzetek:** Paratuberkuloza ali Johnejeva bolezen je danes ena najpomembnejših bolezni pri prežvekovalcih. Povzročitelj bolezni je Mycobacterium avium subsp. paratuberculosis (MAP), ki gostuje pri številnih živalih. Čeprav se bolezen običajno povezuje z govedom in drobnico, so zanjo dovzetni vsi prežvekovalci, vključno z divjimi in eksotičnimi vrstami le-teh. Delež okuženih živali je različen v različnih državah in pokrajinah, na nekaterih področjih je na povzročitelja MAP pozitivnih celo 84,7 % molznih čred. Za paratuberkulozo so najbolj občutljiva teleta, vendar se lahko okužijo tudi telice in odraslo govedo. Novorojenci se običajno okužijo takoj po rojstvu z zaužitjem povzročitelja iz okolice, MAP pa je bil dokazan tudi v mlezivu in mleku asimptomatsko okuženih krav. Čeprav običajno navajajo, da živali ne izločajo povzročitelja pred starostjo leta in pol, je bilo dokazano, da ga lahko izločajo tudi teleta in tako prenašajo okužbo na druga teleta v istem okolju. Velika večina čred se okuži prek nakupa okuženih živali. Prvi klinični znaki so običajno vidni po telitvi kot kronične ali intermitentne driske z obdobji normalne konsistence blata. Pri drobnici driske običajno ni. Subklinično okužene odrasle živali nimajo vidnih znakov okužbe, čeprav so nosilci MAP.

Prognoza je slaba, saj bolezen povzroča hujšanje in izčrpavanje živali, konča pa se s smrtjo. Če ni kliničnih znakov, je treba diagnozo potrditi ali ovreči z laboratorijskimi testi. Za ugotavljanje MAP so na voljo različni testni sistemi, kot so barvanje po Ziehl-Neelsenu, kultura blata in verižna reakcija s polimerazo (PCR) ali ugotavljanje ravni protiteles z metodo ELISA.

Ker je paratuberkulozo težko diagnosticirati in z MAP okužene črede nemogoče ozdraviti, so zelo pomembni preventivni ukrepi za preprečevanje širjenja povzročitelja na neokužene črede. Znotraj črede preprečujemo nadaljnje širjenje bolezni s higienskimi ukrepi, njihov glavni cilj je preprečevanje okužbe telet in mladih živali, pri nakupu pa je treba paziti, da so živali zdrave.

V članku je prikazan pregled načina okužbe, diagnostičnih postopkov in ukrepov za nadzor te bolezni. Osnove smo kombinirali z novimi vidiki sodobnih raziskav in s tem podali natančne in sodobne informacije o paratuberkulozi za veterinarje, ki se ukvarjajo s čredami molznih živali.

**Ključne besede:** govedo, bolezni; paratuberkuloza – diagnostika – epidemiologija; preprečevanje in nadzor; *Mycobacterium paratuberculosis* – patogeneza; govedo; ovce

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**Review Paper**

# AN UPDATE ON UTERINE INFECTIONS IN DAIRY CATTLE

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**Summary:** Inflammation of the uterus, described as acute metritis or chronic endometritis is one of the most common disorders in the postpartum period of dairy cattle. Acute metritis is characterised by reddish-brown and fetid discharge, a body temperature ≥ 39.5°C, often associated with a depressed general attitude, reduced feed intake and decreased milk yield. Bacteria cultured from the uterus are mainly *Escherichia coli*, *Arcanobacterium pyogenes*, and obligate anaerobic species *Fusobacterium necrophorum* and *Prevotella spp*. The recommended therapy of acute metritis is based on a systemic antibiotic treatment without any intrauterine manipulations. Chronic endometritis is defined by the occurrence of purulent or mucopurulent vulvar discharge more than three weeks postpartum. In contrast to acute metritis, chronic endometritis is not associated with an elevated body temperature. For the diagnosis, vaginal inspection via speculum has been demonstrated to be more accurate than rectal palpation. Reproductive performance of affected cows is impaired in the course of lactation. For chronic endometritis, the administration of $PGF_{2\alpha}$ or the intrauterine infusion of Cephapirin is recommended as treatment of choice. In the last years, some new aspects on subclinical endometritis, detected more than three weeks postpartum have been elucidated. In the absence of clinical signs of endometritis, the percentages of polymorphonuclear leukocytes in cytological smears taken from the endometrium or small amounts of fluids in the uterine cavity detected by ultrasound indicate a mild inflammation of the uterus. There is consensus that subclinical endometritis has a significant negative impact on reproductive performance. The treatment of subclinical endometritis, however, is still under discussion. Prostaglandin $F_{2\alpha}$ as well as the intrauterine infusion of Cephapirin or proteolytic enzymes have been tested, but results are not consistent.

**Keywords:** cattle diseases; endometritis – diagnosis; drug therapy; cattle

## Introduction

Inflammation of the uterus, described as acute metritis or chronic endometritis is one of the most common disorders in the postpartum period of dairy cattle. Acute metritis is characterised by reddish-brown, fetid discharge and pyrexia. Chronic endometritis is defined by the occurrence of purulent or mucopurulent vulvar discharge more than three weeks postpartum. The therapy of acute metritis is based on a systemic antibiotic treatment. For chronic endometritis, the administration of $PGF_{2\alpha}$ or intrauterine antibiotics is recommended. In the last years, some new aspects on subclinical endometritis, detected by cytological examinations and the use of ultrasound have been elucidated. There is consensus that subclinical endometritis has a significant negative impact on reproductive performance.

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## Acute metritis

Acute metritis is also referred to as postpartum metritis, toxic puerperal metritis or septic metritis and occurs within the first 10 days after parturition. It is characterised by fetid, watery, and reddish-brown to purulent vulvar discharge and an elevated body temperature ≥39.5°C (1). Referees on which body temperature can be regarded as fever range from 39.2°C (2) to 39.7°C (3). The palpation of the uterus per rectum reveals an enlarged and flaccid uterus. Acute metritis is often associated with a depressed general attitude, reduced feed intake and decreased milk yield.

Risk factors for acute metritis were categorised by Sheldon and Dobson (4) into uterine damages (stillbirth, dystocia, twins, cesarean section, retained placenta, delayed uterine involution), metabolic conditions (milk fever, ketosis, left displaced abomasum) and the balance between pathogenicity and immunity (disruption of neutrophil function, type of bacterial flora, proges-

terone and glucocorticoide administration, early formation of a corpus luteum, level of hygiene).

*Infection of the postpartum uterus and uterine defense mechanisms*

The infection and to some extent the inflammation of the uterine wall during and after parturition must be accepted as a physiological process (5). Pathogenic species for metritis isolated from the uterine cavity are *Escherichia (E.) coli*, *Arcanobacterium (A.) pyogenes*, and obligate anaerobic species *Fusobacterium (F.) necrophorum* and *Prevotella spp.* (5, 6). Beside the quantity and quality of bacteria in the uterus, the efficiency of uterine defense mechanisms determines the severity of metritis. The uterine defense mechanisms consist of anatomical and physical barriers, i.e. the vulvar and cervical closure as well as the cell-mediated and humoral immune systems. The initial cellular response to an infection of the uterine wall is an influx of polymorphonuclear leukocytes (PMN) and macrophages. Immunoglobulins and opsonins are released from the endometrium (7, 8).

*Antibiotic treatment of acute metritis*

An ideal treatment of metritis should eliminate bacteria from the uterine cavity and the subendometrial layers without inhibiting uterine defense mechanisms. It should provide optimal reproductive performance in the current lactation, and not cause economic losses by milk withdrawl. In the last years, strategies for the therapy of acute metritis were focused on a systemic antibiotic treatment. The efficacy of a systemic administration of penicillin, oxytetracycline, or ceftiofur in cows with acute metritis or retained fetal membranes, often associated with acute metritis, has been demonstrated in several studies with regard to clinical cure rates and reproductive performance (2, 3, 9, 10, 11). Ceftiofur concentrations exceeded the minimum inhibitory concentration ($MIC_{90}$) for *A. pyogenes*, *E. coli*, *F. necrophorum* in blood and endometrial tissue within two hours after administration (12).

The efficacy of a local antibiotic treatment (solutions or uterine pills) is a controversial issue in the literature (2, 5, 9, 11). A recent multi-located field trial on cows with retained fetal membranes has shown that the additional use of antibiotic pills had no benefits compared with the sole systemic antibiotic use of ceftiofur (11). Negative interactions between antibiotic drugs and the uterine environment, the inhibition of the uterine defense mechanism by irritating drugs, solutions and antibiotics, and a questionable therapeutically efficacy of antibiotics within the inflamed uterine wall and the oviducts are some reasons to reject a local treatment (5). The application of higher dosages of antibiotic drugs to reach a $MIC_{90}$ in the uterine wall increases the risk for antibiotic residues in milk and is not in accordance to legal drug regulations and the guidelines for a prudent use of antibiotic drugs.

*Additional treatment*

Clinical trials on the application of non-steriodal antiinflammatory drugs in the early postpartum period did not show beneficial effects on clinical cure rates (13). The use of estradiol to stimulate uterine motility and immunity is not approved for uterine disorders in the European Union. In addition, recent studies demonstrated no positive effects on the prevention or treatment of acute metritis (3, 10). In severe cases of acute metritis, a rehydration therapy applied systemically and/or by drenching seems to be helpful to wash out toxins.

## Chronic endometritis

Chronic endometritis is also referred to as clinical endometritis and is characterised by the presence of mucopurulent or purulent exudate in the vagina three weeks or more after parturition (1). In contrast to acute metritis, chronic endometritis is not associated with elevated temperature and depressed general attitude. Several methods for diagnosing endometritis have been established, including adspection of the vulva, perineum and the tail, combined with manual palpation of uterus and cervix per rectum, and/or vaginal inspection. Clinical findings by *rectal palpation* of the uterus are asymmetric uterine horns, thickened uterine wall, palpable presence of fluid (5), and a cervical diameter of more than 7.5 cm (14). V*aginal inspection* via speculum, however, is more accurate than rectal palpation (5, 14). Rectal palpation as well as vaginoscopy are indirect diagnostic methods and can not verify the inflammation of the uterus itself. *Uterine swabs* for microbiology as a routine diagnostic tool for chronic endometritis is not practical and associated with high costs (5). Common bacteria isolated from cows with chronic endometritis are *A. pyogenes*, *E. coli,* as well as *F. necrophorum* and *Prevotalla spp* (1, 5). Infections with *Chlamydophila spp* can also result in chronic endometritis (15). Positive

findings, however, provide only evidence for an infection but not for endometritis. *Biopsy* of the uterine endometrium provides informations about local histological alterations. The manipulation itself, however, can impair subsequent fertility of the tested cows (16).

Numerous attempts were made to categorize chronic endometritis. An endometritis scoring by Williams et al. (17) is online available at

http://www.rvc.ac.uk/AboutUS/Staff/sheldon/ResearchInterests/Gallery/index.cfm. Cloudy discharge and clear mucus with flakes of pus in the absence of an enlarged uterus can be regarded as signs for mild endometritis. An enlarged, fluid-filled uterus accompanied with an enlarged cervix and purulent discharge indicates a severe endometritis (17). Pyometra is defined as an enlarged fluid-filled uterus without any visible discharge and the presence of a corpus luteum (1, 5).

The best time for diagnosis is controversial. Some studies have shown a tendency to self-recovery from endometritis within the postpartum period. Therefore it seems rational that the diagnosis should be performed about three to four weeks after calving (14, 18).

The impact of chronic endometritis on reproductive performance is characterised by decreased service and conception rates, and consequently by prolonged days to first service and days open. The number of cows pregnant is decreased while the risk for culling is increased (5, 19, 20, 21).

*Treatment of chronic endometritis*

In general, the treatment of chronic endometritis is based on two different strategies, i.e. an intrauterine treatment with antibiotics or a systemic treatment with prostaglandin $F_{2\alpha}$ (4, 19, 22). It has been discussed whether cases of mild endometritis have to be treated at all (18, 19). Studies on subclinical endometritis, however, indicate to treat all cows with any signs of endometritis (23, 24, 25). The central mechanism of a treatment with $PGF_{2\alpha}$ and its analogues is the luteolytic activity of $PGF_{2\alpha}$, followed by onset of estrus (5). The myometrium contracts and uterine fluids such as pus can pass through an open cervix. The influx of PMN into the mucosa increases and mucus containing immunoglobulins is produced (8).

Some studies described an intrauterine antibiotic treatment with cephapirin as equally efficacious or superior to the application of $PGF_{2\alpha}$ (20, 21, 26). With regard to a prudent use of antibiotic drugs in food producing animals and to minimize the risk of provoking antibiotic resistance, the application of antibiotics should be limited to cases that can not be controlled by $PGF_{2\alpha}$.

The uterine infusion of antiseptics has been routinely used in veterinary practice in several countries for many years. Intrauterine infusions, however, failed to show positive effects on subsequent reproductive performance (19, 22, 27), but have been described as detrimental on uterine defense mechanisms and the epithelium of the oviducts (27). Studies on intrauterine applications of herbal extracts (22) or proteolytic enzymes (28) provided some promising approaches, but failed to give convincing results compared to the application of $PGF_{2\alpha}$.

*Subclinical endometritis*

In the absence of clinical signs of chronic endometritis, alterations in the uterine lumen or uterine wall can be defined as subclinical endometritis. Some recent studies described the diagnosis and treatment of subclinical endometritis. *Ultrasonography* as a non-invasive method visualizes small amounts of fluid in the uterine lumen (24, 29). False positive findings might result from clear mucus in the uterus appearing during estrus. Therefore, the ovaries should be scanned as well to define the stage of the estrus cycle. *Endometrial cytology* can be performed by flushing the uterus to obtain endometrial cells or taking samples with a cytobrush from the endometrium. The percentage of PMN in the cytological preparation provides information on the presence of subclinical endometritis. The threshold value for PMN varies between authors from 5 to 18% (23, 24, 30). It has been demonstrated that cows with subclinical endometritis have a depressed reproductive performance in the current lactation (23, 24). For the treatment of subclinical endometritis, intrauterine infusions with cephapirin as well as the administration of $PGF_{2\alpha}$ have been recommended (25). Other studies, however, did not confirm the efficiency of this treatment (30, 31).

The challenge for veterinarians is an accurate diagnosis and efficacious treatment of cows with acute, chronic and subclinical endometritis. The efficacy of a treatment must be evaluated with regard to cure rate and subsequent reproductive performance. The objective of herd health management must be the prevention of metritis by adequate feeding, hygienic calving conditions and careful obstetrical assistance.

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## NOVOSTI S PODROČJA MATERNIČNIH OKUŽB PRI KRAVAH MOLZNICAH

M. Drillich

**Povzetek:** Vnetja maternice, ki jih opisujemo kot akutni metritis ali kronični endometritis, so ena izmed najpogostejših motenj v poporodnem obdobju pri kravah molznicah. Za akutni metritis so značilni rdečerjav smrdljiv izloček, telesna temperatura ≥ 39.5 °C, ki jo pogosto spremlja splošna potrtost živali, zmanjšana ješčnost in zmanjšana proizvodnja mleka. Iz materničnega brisa izoliramo predvsem bakterije vrste *Escherichia coli*, *Arcanobacterium pyogenes* ter obligatne anaerobe *Fusobacterium necrophorum* in *Prevotella spp.* Priporočeno zdravljenje akutnega metritisa temelji na sistemskem zdravljenju z antibiotiki brez kakršnih koli posegov v maternico. Kronični endometritis definiramo ob pojavu gnojnega ali sluzasto-gnojnega izcedka, ki se pojavi po tretjem tednu po porodu. V nasprotju z akutnim metritisom kroničnega ne spremlja povišana temperatura. Vaginalni pregled s spekulumom se je izkazal za boljše diagnostično sredstvo kot pa rektalni. Med laktacijo se reprodukcijska sposobnost prizadetih krav zmanjša. Pri kroničnem endometritisu se priporoča aplikacija prostoglandina $F_{2\alpha}$ ($PGF_{2\alpha}$) ali intrauterina infuzija cefapirina. Zadnja leta so prinesla tudi nekaj novih pogledov na subklinični endometritis, ki ga zaznamo po treh tednih po porodu. Kliničnih znakov endometritisa ni, vendar v citoloških brisih endometrija ali v majhni količini tekočine v maternični svetlini (ki jo zaznamo z ultrazvokom) lahko ugotovimo odstotek polimorfonuklearnih levkocitov, ki kažejo na blago vnetje maternice. Sprejeto je splošno mnenje, da subklinični endometritis izrazito negativno vpliva na reprodukcijsko sposobnost, o načinih zdravljenja pa se še razpravlja. V poskusih so uporabili aplikacijo $PGF_{2\alpha}$ in intrauterino infuzijo cefaprina ali proteolitičnih encimov, vendar se rezultati teh testov ne ujemajo.

**Ključne besede:** govedo, bolezni; endometritis – diagnostika; zdravila, zdravljenje; govedo

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**Review Paper**

# INHERITED DISORDERS OF CATTLE: A SELECTED REVIEW

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**Abstract:** In this paper, the authors summarize the characteristics of the most important inherited disorders they have encounteredfirst hand.
For some of the diseases discussed, the genetic origin has been definitively stated. For others, it is still only a hypothesis which has yet to be confirmed. For all of them, the authors emphasize the importance of identififying and reporting them to diagnostic centres. At the moment in Italy, the authors are trying to develop a program to identify carriers of an undesirable pathological character and to increase relative clinical and pathological knowledge.

**Key words:** hereditary diseases; central nervous system diseases – genetics; skin diseases – genetics; bone diseases – genetics; cattle

## Introduction

Strong inbreeding in the bovine population has increased the risk of the occurrence of genetic diseases. In fact, the wide use of only a few elite sires has enhanced the probability of the coupling of two mutated recessive genes in the genotype of an animal.

One of the most important problems in controlling the genetic diseases is that, once the disorder has finally been discovered, the allelic frequency of the recessives might have already reached high values in the population of the affected breed. In fact, it usually turns out that a genetic disease reveals itself many years after the mutation has occurred, corresponding to the time that the male and female descendants of the original carrier are mated. In the meantime, the allele might have been widely spread throughout the bovine population.

For this reason it is very important that, as early as possible, the phenotype associated with a physiological abnormality, biochemical defect or enzyme deficiency be attributed to a mutated homozygous genotype.

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Conditions alerting the investigator to the fact that an abnormality is likely genetic in nature are:
1) it is more common in a group of related animals;
2) it is observed during all seasons of the year and in different geographic locations;
3) it appears more frequently as the level of inbreeding increases.

Whatever the causes, the first step in reducing the incidence of any defect is an accurate clinical and pathological description. The reporting and, possibly, the referring of any suspected case to diagnostic centres is therefore an indispensable step towards improving the possibility of recognition. At the moment in Italy, we are trying to develop a program to identify the carriers of an undesirable pathological character and to increase relative clinical and pathological knowledge so that future cases can be better diagnosed and possibly prevented.

This paper reviews the characteristics of the most important inherited disorders we have encountered first hand. The diseases discussed are classified according to the principal body system affected.

Bibliographic references are limited to part of the papers related to genetic diseases written by the authors together with the most significant and recent articles in the literature.

## Inherited central nervous system diseases

*Spinal Muscular Atrophy* (1, 2, 3, 4, 5, 6, 7)

Spinal Muscular Atrophy (SMA) is a progressive lethal disease reported mainly in advanced backcrosses between American Brown-Swiss and European Brown cattle breeds but also described in Holstein-Friesian calves. It represents the most worrisome concern for Brown breeders' associations.

The condition is characterized by severe muscular atrophy, progressive quadriparesis, and sternal recumbency. The initial signs - symmetric weakness of the rear legs, locomotive difficulties and slight dyspnoea - appear at 3-4 weeks of age. The course of the disease is progressive, and the calves become increasingly weaker and progress to paraparesis and finally tetraparesis (Fig. 1). Animals usually look alert and have a good appetite and a normal suckling reflex. Urination and defecation are in the physiological range. The symptoms are quite similar to white muscle disease (nutritional muscular dystrophy). Death occurs after 2-4 weeks, usually as a consequence of respiratory failure due to atrophy of the respiratory muscles. Occasionally, calves are unable to stand up from the moment of birth. Histo-pathologically, the condition is mainly characterized by muscle fibre atrophy and axonal degeneration of the spinal cord as well as neuronophagia and degeneration and loss of motor neurons in the grey matter of the ventral horns (especially in the brachial and lumbo-sacral regions); furthermore, severe vacuolar degeneration in the midbrain and central motor cortex can be observed. Neuron degeneration seems to originate in unrestrained apoptotic processes initiated during foetal development.

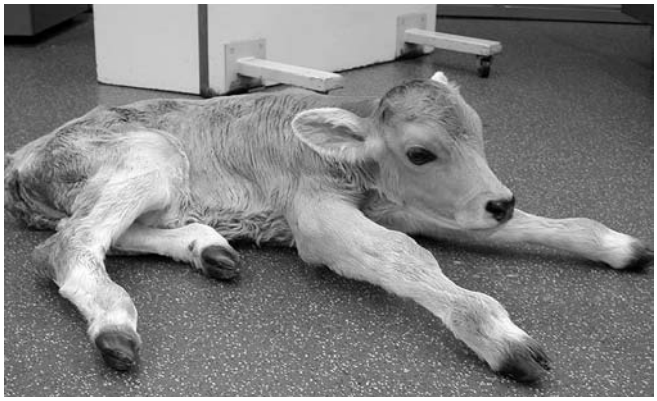

**Figure 1:** Two-week-old Brown calf affected by Spinal Muscular Atrophy; the calf is weak and not able to maintain the quadrupedal stance. Forelegs are typically maintained extended forward. Note the muscular atrophy of the hindquarters

Bovine-SMA is inherited as an autosomal recessive disorder and its gene has been mapped to the distal part of Chromosome 24. Most of the cases reported can be traced back to an American Brown Swiss bull named "Meadow View Destiny".

At present, marker-assisted tests are available in order to detect carriers of this undesirable gene.

*Spinal Dysmielination* (8, 9, 10, 11,12, 13)

Spinal Dysmielination (SDM) is another congenital and genetic neurological disorder mainly affecting Brown or cross-bred calves upgraded with American Brown Swiss.

Affected animals have congenital recumbency (contrarily to SMA) and, for the most part, lie in a lateral position with a slight to moderate opisthotonos (Fig. 2). Rear limbs are held in extension and. on pressuring the interdigital skin, they react by stretching or kicking. The hind limbs also remain typically extended if calves are able to maintain the sternal position. Although the animals do not try to rise, they are attentive to their surroundings. Main reflexes, appetite, faeces and urine delivery are normal. Affected calves usually die or are euthanized during the first week of life.

**Figure 2:** Two-day-old Brown calf affected by Spinal Dysmielination; the calf is lying in a lateral position with opisthotonus

Histopathologically, the disorder is mainly characterized by bilateral symmetrical dysmielination in the white matter of the spinal cord (gracile funiculus, dorsolateral spinocerebellar tract, sulcomarginal tract), especially at the level of cervical intumescence. Typically, the submeningeal areas have a more pronounced dysmielination than the deeper parts. Moreover, the number of axons within the affected tracts is reduced. Myelination of the dorsal and ventral nerve roots appears normal.

Similarly to bovine-SMA, SDM is an autosomal recessively-inherited defect. There is evidence that SDM might be traced back to an American Brown Swiss bull named "White Cloud Jasons Elegant" born in 1966.

A marker-assisted test based on five markers has recently been developed in order to detect carriers of this undesirable gene. It is however limited to some genetic lines.

*Bovine Progressive Degenerative Myeloencephalopathy ("Weaver" Syndrome)* (14, 15, 16)

Bovine Progressive Degenerative Myeloencephalopathy (BPDM) is an inherited disorder of purebred Brown cattle characterized by progressive bilateral hind leg weakness and ataxia, resulting in a weaving aspect of the gait.

Clinical synptoms become apparent at about 6-8 months of age and slowly increase in severity until the animals become unable to rise. Paresis and ataxia are due to proprioceptive deficits involving all four limbs, although the hindlimbs usually appear worse than the forelimbs. If turned quickly or stimulated to run, the hindquarters tend to lose balance and the animals fall laterally (Fig. 3). The same happens if lateral pressure is applied anywhere above the stifle or at the level of the hip. The deficit increases slowly in affected animals and invariably becomes recumbent.

**Figure 3:** Eighteen-month-old Brown heifer affected by "Weaver syndrome"; stimulated to run, the animal tends to lose balance and falls laterally

Histopathologically, the lesions are characterized by axonal degeneration and vacuolation of the white matter of the spinal cord and degenerative changes or numeric reduction of the Purkinje cells in the cerebellum.

The U.S. sire "Nakota Destiny Dapper" and its sons "Target" and "Matthew" were responsible for the diffusion of BPDM. The defect has been mapped through linkage analysis to bovine Chromosome 4. A strong selective advantage for milk production has been demonstrated in BPDM-carriers. This was the reason which caused the increase in the frequency of the defective gene. An official DNA marker test allows control of the gene frequency effectively enough, without removing identified BPDM-carriers from service.

*Spastic Paresis* (17, 18, 19, 20, 21, 22)

With the term Spastic Paresis, we recognize a sporadic neuromuscular disease of cattle clinically characterized by a hyperextension of the rear limbs ("straight hock") due to a contraction of the muscles which form the Achilles tendon (Fig. 4). Signs of Spastic Paresis usually appear at the age of 3 to 8 months, although these signs may appear when the calves are only a few weeks old as well. More unusual are the cases of later onset, as late as 3 years (Fig. 5).

**Figure 4:** Seven-month-old male Romagnola animal affected by Spastic Paresis; note the hyperextension of the hock and the "pendulum" movement of the right hind limb

**Figure 5:** Six-year-old Holstein cow affected by Spastic Paresis; the left hind leg remains completely raised from the ground and extended backwards

In the initial stage, the most remarkable finding is the hyperextension of the hock with an increase of the tibiotarsal angle ("straight hocks"). The condition has a progressive but not predictable course over a period of a few weeks or months; the straightness of the limb become more severe and the calcaneus tends to be drawn to the tibia so that it is possible to observe a wrinkling of the skin corresponding to the distal part of the Achilles tendon. The stifle can however be easily flexed and, in this way, tremulous contractions and further rigid extension of the limb can be provoked. The affected animal has a stiff gait and moves without normal hock flexion.

Later on, the leg is held so that the foot just touches the ground with the toe or it remains completely raised from the ground and extended backwards. In these cases, the animal uses only three legs to walk; the severely affected leg is held permanently in extension and contractural fits cause a typical "pendulum" movement, with most motion occurring at the coxofemoral joint. This sign is most evident immediately after standing up when it is also possible to observe an arching of the back and elevation of the tailhead.

In the majority of cases, only one leg is affected: if both limbs are involved, the animal bears weight alternatively on each leg.

It is worth noting that, in recumbency, the animal is completely normal. Moreover, the tone of the affected muscles is normal as has been confirmed by electromyographic studies.

Spastic Paresis is caused by a spastic contraction of the antigravitational, foot extensor muscle group, especially the gastrocnemius and the superficial digital flexor. Other muscles such as biceps femoris, semitendinosus, semimembranosus, quadriceps femoris and adductor muscles can also be involved. The contraction of the quadriceps femoris characterizes the recently described atypical form of Spastic Paresis of the femoral quadriceps, first observed in Belgian White Blue calves but then also observed in Romagnola animals. In these cases, the affected hind leg shows a swaying movement in anterior direction.

The muscular spasticity should be attributed to hyperactivity of the myotatic reflex ("stretch reflex"). The dysfunction lies primarily in the gamma-pathway which means in an anomalous action of the gamma-motor neurons. On the basis of this pathogenetical hypothesis, it is nevertheless still unknown whether the overstimulation of the gamma-pathway is due to intrinsic hyperactivity of the gamma-motor neuron or to a lack of inhibitory mechanisms; in the latter case, a defect of the regulatory descendant pathway arising from the red nucleus (rubrospinal pathway) or from the lateral vestibular nucleus (vestibulospinal pathway) could play an important role.

Although the hereditary contribution appears unquestionable, up to now, it has not been possible to definitively determine the mode of inheritance (dominant or recessive) or the entity of penetrance of the single or multiple genes responsible. It is supposed by most authors that the mode of inheritance could be recessive with low or incomplete penetrance. It should therefore be assumed that environmental (plant toxicity?), nutritional (trace element deficiency = Mn, Ca, P, Cu, Zn, Co, I, Se?; vitamin deficiency = vit. A?), metabolic (Cu/Zn imbalance?) or individual factors can play an important role in the appearance and expression of the disease.

Many types of therapy have been suggested, including tenotomy of the gastrocnemius tendon and neurectomy of the branches of the tibial nerve supplying the gastrocnemius muscle. Our experience is limited to the total neurectomy of the tibial nerve which has shown interesting results but not a complete recovery.

*Spastic Syndrome* (23, 24, 25)

Spastic Syndrome ("crampiness", "Krämpfigkeit") is a chronic condition occurring in adult cattle. It is a particular problem in the mature bulls maintained in artificial insemination centres but can also affect cows in a recurrent or in a progressive form. Animals of several breeds can be affected. The disease is characterized by intermittent bilateral spasms of the skeletal muscles of the pelvic girdle, including the muscles of the rump. Each spasm is accompanied by kyphosis which is often terminated by a tremor of the hindquarters (Fig. 6). During the attack, one hind

**Figure 6:** Three and a half-year-old Holstein cow affected by Spastic Syndrome; note the kyphosis

leg (usually always the same) may lift laterally in partial flexion. The intensity and duration of the spasms progressively increase over time.

There is a paucity of literature on Spastic Syndrome and the few reports which included morphological examinations failed to reach significant results and concluded that the syndrome is a type of idiopathic or true muscle cramps.

Regarding the aetiology, there is evidence which suggests a recessive mode of inheritance but with incomplete penetrance.

There is no specific treatment for the condition; we personally recommend the use of analgesic drugs.

## Inherited congenital skeletal malformations

*Craniofacial defects*

Some of the literature lists closure defects of the lip (cleft lip) and/or the palate (cleft palate – Fig. 7) as genetic defects, likely polygenic, but more data are needed to confirm this hypothesis. In any case, it should be mentioned that these facial defects (especially cleft palate) constitute a relatively frequent congenital anomaly which may be part of a complex congenital malformations in varying degrees.

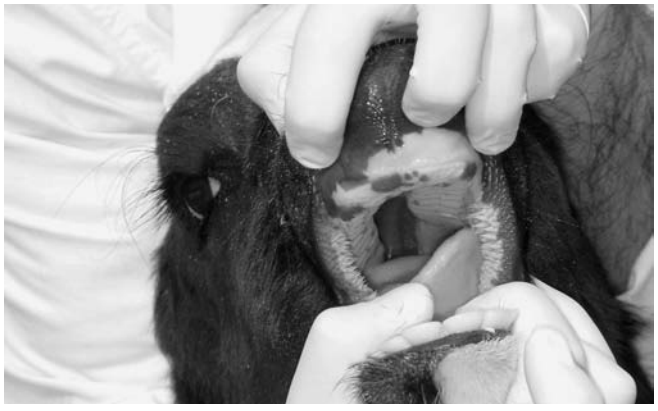

**Figure 7:** 40-day-old calf affected by cleft palate

Another relatively common, more or less pronounced, congenital anomaly is short lower jaw (brachygnatia). Also for this defect, an inherited origin is suggested, especially for Simmentals and, more recently, Brown breeds.

*Chondrodysplasia and " Congenital Paunch Calf Syndrome"* (26, 27, 28, 29, 30)

Chondrodysplasia is a disturbance of endochondral ossification leading to disordered bone development. Although several types of chondrodysplasia are known, the most common form in Italy is the so-called "Bulldog calf" of the Romagnola breed (Fig. 8). It is characterized by a flat head with a short nose and sloping forehead and by short and stumpy limbs. Severe affected calves live only a few months.

**Figure 8:** Six-month-old Romagnola animal affected by achondroplasia; note the facial deformity

The bone growth disturbances involve a defect of cartilage growth which hinders the normal lengthening of the long bones. The chondrocytes do not undergo physiological differentiation so they cannot fulfil normal endochondral ossification in osteogenesis.

More recently, we have reported another form of achondroplasia in Romagnola calveswhich we have called "Congenital Paunch Calf Syndrome" because, besides facial defects, another main clinical feature is abdominal distension and also because this is the name used by farmers when they report the affected animals.

Facial deformities are characterized by a shortened and flattened face and, in some cases, by an enlarged head. contrarily to other achondroplastic defects, in this case, limbs only rarely show a disproportionate shortness. Cleft palate is a frequent accompanying finding.

The most characteristic symptom of these calves, however, is an enlarged and floating abdomen, denoting considerable abdominal effusion (Fig. 9). Moreover at necropsy, the animals usually show marked subcutaneous oedema, especially in the ventral part of the abdominal wall. Different quantities of ascitic fluid (in some cases up to 10 litres) are present in the abdominal cavity. The liquid ranges from yellow to red, with different grades of turbidity.

Another characterizing aspect is moderate to severe diffuse hepatic fibrosis, associated with the presence of hepatic cysts, containing serous or reddish fluid. Microscopic examination of the liver reveals an extensive distortion of lobular

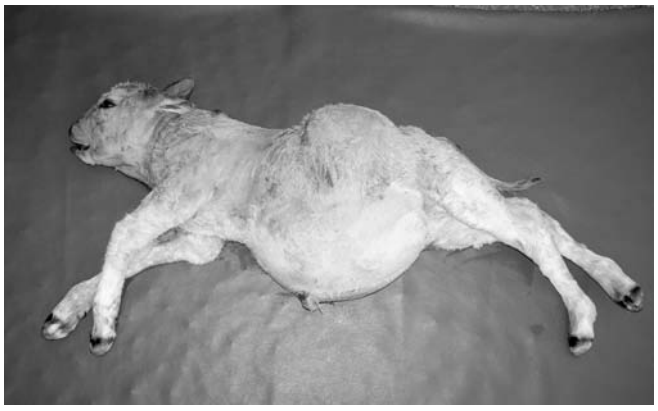

**Figure 9:** Stillborn Romagnola calf affected by the so-called "Congenital Paunch Calf Syndrome"; note the enlarged and floating abdomen (denoting a considerable abdominal effusion) and the facial deformities (shortened and flattened face)

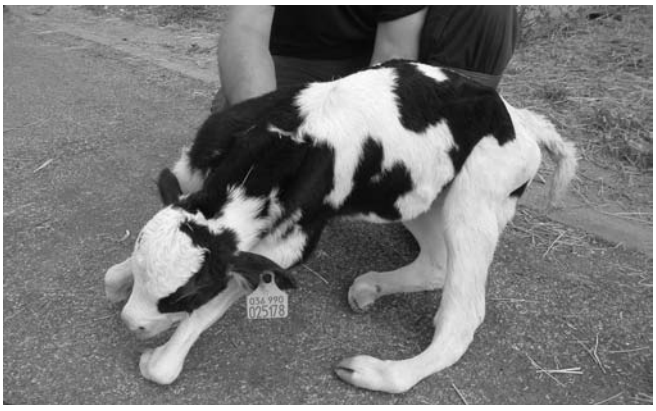

**Figure 10:** Two-day-old Holstein calf affected by Complex Vertebral Malformation; despite repeated attempts to stand up, the calf is unable to do so. If supported by assistants, its feet rest on the dorsolateral face of the pastern on the ground; the head hangs down between the forelimbs

architecture by widespread fibrosis in periportal areas and around the centrolobular veins. In some lobules, the fibrosis extends to the perisinusoidal spaces.

Cardiac malformations, such as atrial and interventricular septal defects, and patent ductus arteriosus can be observed.

At the moment, we have not found any familial line which can be considered responsible for the inheritance of the defect; nevertheless, a genetic cause is strongly suspected.

*Complex Vertebral Malformation* (31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41)

At present "Complex Vertebral Malformation" (CVM) represents the most worrisome hereditary concern for Holstein breeders. This defect is clinically characterized by the following phenomenological triad: (a) reduced body size; (b) symmetrical arthrogryposis; (c) malformations in the cervical and/or thoracic vertebral column (especially shortened neck and scoliosis). Homozygous animals usually die during pregnancy, are premature or stillborn, or die shortly after birth. Besides the aforementioned clinical triad, live calves might be alert and show a suckling reflex and appetite. They are unable to stand up (Fig. 10), lying down in a flat position with extended limbs (frog-like decubitus).

Clinical diagnosis is confirmed by radiographic examination of the vertebral column which shows multiple vertebral anomalies including hemivertebrae, fused and misshapen vertebrae and ribs, and scoliosis affecting mainly the caudal cervical and the thoracic regions (Fig. 11).

A complex malformation of the heart, characterized by atrial and/or interventricular septal defects, and patent ductus arteriosus might accompany the skeletal malformations.

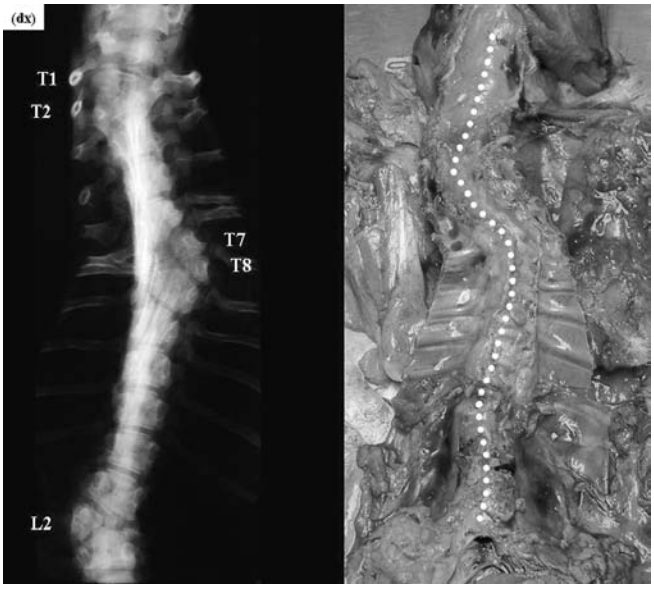


**Figure 11:** X-ray and anatomical dissection of the same calf as in Fig. 10 = on the left: note the presence of hemivertebrae in the thoracic (T1, T2, T7, T8) and lumbar (L2) regions; on the right: note the evident S-shaped deviation of the vertebral column at the level of the thoracic and lombar tracts (scoliosis)

CVM stems from a simple autosomal recessive allele which can be detected by a DNA-PCR based test.

Interesting recent analysis has demonstrated that a different expression of the foetal CVM phenotype might directly influence several fertility traits, such as non-return rates for cows, calving

frequency after a first insemination and the interval between insemination and the next calving; according to recent research, 16% of CVM-affected embryos die within the first 56 days of pregnancy, and up to 45% and 77% of CVM-affected foetuses die before the 150$^{th}$ and the 260$^{th}$ gestation-day, respectively. The frequency of calving resulting in a live-born calf 260-300 days after first insemination has been calculated to be reduced by 93% if the foetuses had the CVM phenotype, therefore deducing that only 7% of CVM-affected calves survive the gestation period.

Unless the malformation is severe, the vertebral defect might be overlooked during routine examination of the aborted foetuses or stillborn calves, and the same may be said of neonates apparently affected by only arthrogryposis and incapable of assuming the quadrupedal stance.

The embryonic/foetal mortality rate might also be biased by the observed rate of heat, the culling of pregnant cows and embryonic/foetal mortality without any connection to CVM. All these situations negatively influence the perception of the presence of the defect in the herd and, therefore, do not allow the exact estimation of the economic loss related to foetal and near-term deaths associated with the defect.

Retrospective evaluation of the familial occurrence has demonstrated that the former elite U.S. Holstein Carlin-M-Ivanhoe Bell should be considered one of the biggest spreaders of the disease. It very probably received the defective allele from its father, Penstate Ivanhoe Star. It should be mentioned that the same family of sires has been recognized as te carrier of another recessive gene defect, Bovine Leukocyte Adhesion Deficiency (BLAD). However, the two gene defects are not linked and are inherited independently.

*Arachnomelia* (42, 43, 44, 45, 46, 47, 48, 49)

Arachnomelia ("spider-legs") is a congenital abnormality of the skeletal system giving the animal a spidery look, and reported both in Simmenthal and Brown calves.

The most important pathologic findings are: facial deformities (i.e. brachygnatia inferior and concave rounding of the dorsal profile of the maxilla), bone dolichostenomelia, angular deformities in the distal part of the hind legs (Fig. 12), muscular atrophy and cardiac malformations. The bones of the legs appear to be more fragile than normal and spontaneous fracture during calving may injure the dam.

Although we failed to find precise information in the literature, the pathogenesis of the disease

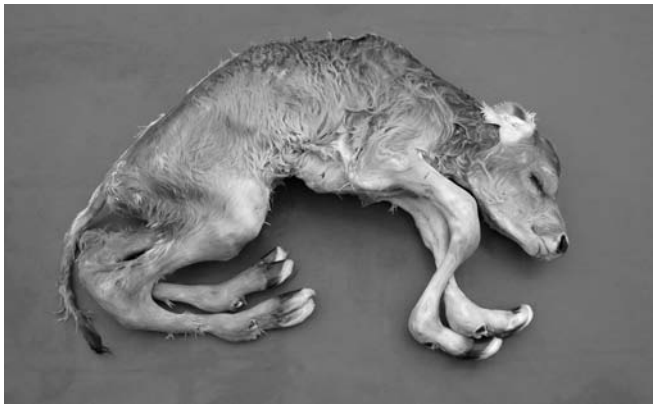

**Figure 12:** Stillborn Brown calf affected by Arachnomelia; note the abnormal length and thinning of all legs (dolichostenomelia). Forelegs show severe angular deformities in their distal part

seems to overlap that of Marfan Syndrome in human medicine (Arachnodactylia); in this context, a defect in the metabolism of the connective tissue is involved. However, the clinical findings recorded in calves affected by Arachnomelia usually differ from the typical picture of human "Marfan patients" (dolichostenomelia with high fragility of the long bones, defects of the heart and main arteries and ectopia lins) and, for this reason, we think that the clinical identification between bovine Arachnomelia and human Marfan Syndrome is inappropriate. Moreover, contrary to the almost undisturbed vitality of human patients, bovine Arachnomelia has a rapidly lethal course.

It should be kept in mind that true Bovine Marfan Syndrome, more closely resembling human Marfan Syndrome, has also been described in cattle.

As far as the aetiology is concerned, although it has not been possible to find candidate genes until now, the condition is attributable to a simple autosomal recessive inheritability. The U.S. sire "Novic Lilasons Beautician" was responsible for the diffusion of the defect . At the moment, there is neither a chromosomal nor a biochemical test to detect the carriers of this defect.

*Short Spine Lethal* (50, 51, 52, 53, 54, 55)

Short Spine Lethal is a rare skeletal malformation externally characterized by a marked and disproportionate shortening of the vertebral column associated with a normally developed head and legs (Fig. 13). Shortening of the neck might be very pronounced giving the impression that the head is fixed to the chest. Misalignment of the teeth and mandibular hypoplasia, resulting in

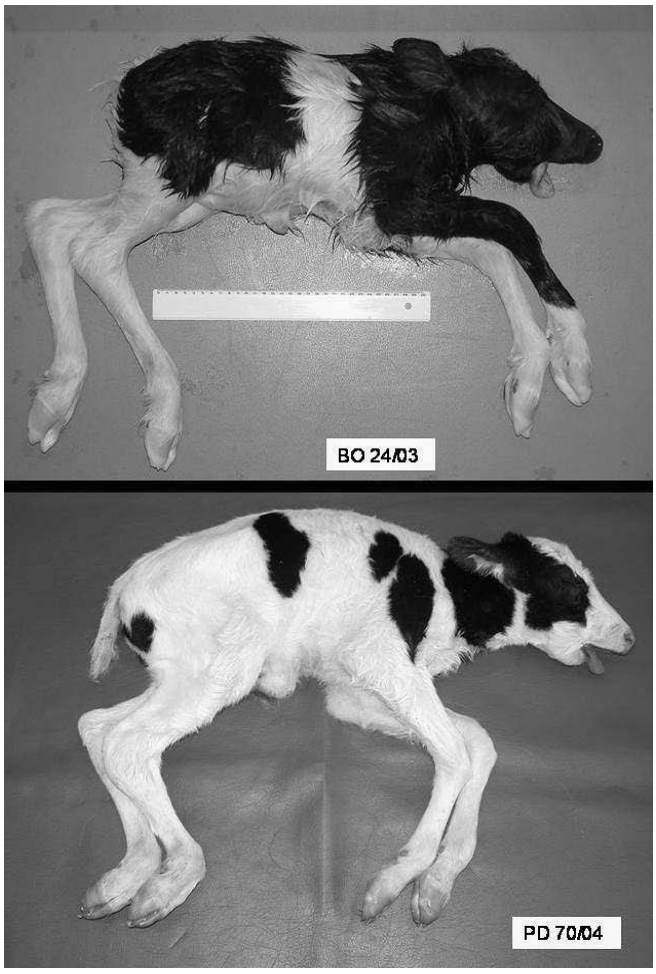


**Figure 13:** Stillborn Holstein calves affected by Short Spine Lethal. In both animals the trunk was disproportionately short for the body size and legs. Note the mandibular hypoplasia and the protrusion of the tongue. Both calves were traced back to the same sire, one of the breeding lines most recently used in Holsteins in Italy

protrusion of the tongue from the oral cavity, might be observed. Despite being full term, calves might present a strong reduction in body weight.

External conformation is due to several spinal malformations, including fusion and/or variations in the number of vertebrae, abnormal vertebral body size and shape, and misalignment of vertebral segments, mainly involving the cervical and thoraco-lumbar tract.

Visceral malformations involving the urogenital, gastrointestinal, and cardiovascular systems may also be found.

In all cases published, the occurrence of common ancestors has suggested a genetic aetiology. Unfortunately, due to the limited number of cases, evaluation of the mode of inheritance is difficult.

*Perosomus acaudatus/elumbis* (56, 57, 58, 59)

Partial agenesis of the vertebral column constitutes a relatively common occurrence and can be limited to the coccygeal tract (Perosomus acaudatus, Brachyury) or also involve the sacral and/or lumbar tract (Perosomus elumbis). Although evidence of inheritance has been reported in the past, more information is needed to confirm the genetic hypothesis.

The simple lack of the coccygeal vertebrae (Perosomus acaudatus) is compatible with life and animals can also carry out pregnancy without any problem. In more extended failures of vertebral development (Perosomus elumbis), the back of the lumbosacral region lacks a rigid skeletal support, and the hindquarters, strongly underdeveloped and possibly paralyzed, are linked to the rest of the body only by soft tissue. In these cases, calves are shorter than normal and cannot maintain a quadrupedal stance or use their hind legs at all (Figs. 14 and 15).

**Figure 14:** Brown calf affected by Perosomus elumbis; note the brachygnathia of the lower jaw and the shortened torso

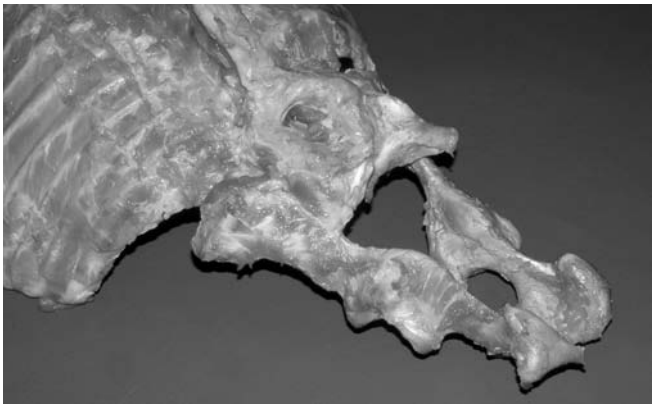

**Figure 15:** Anatomical dissection of the same calf as in Fig. 14; the lumbar, sacral and coccygeal vertebrae were entirely lacking

The skeletal anomaly is often accompanied by other endocavitary malformations (e.g. colon/rectum/anal atresia with a blind-ending-sac filled with mucoid faeces and protruding at the level of the sacral region, renal agenesis, cardiac anomalies) which make the situation incompatible with life. The spinal cord ends in a blind vertebral canal.

Malformation or improper migration of the neural tube during the tail-bud stage, accompanied by partial agenesis of the caudal spinal cord, seems to be the cause of this abnormality.

*Syndactyly (Mule Foot)* (60, 61, 62, 63)

Fusion or non-division of the digits constitutes a well known hereditary defect in Holstein cattle but has been described in a number of other cattle breeds. The lesion is primarily osteological in nature and consist mainly in synostotic phalanges; more proximal limb structures can be affected as well, such as metacarpal and metatarsal III and IV bones. The syndactylous hoof has the aspect of a truncated cone with the base at the coronary seam. In Holstein cattle, Mule Foot has been confirmed as a simple autosomal recessive inherited defect; the gene responsible is located on the telomeric end of chromosome 15. An incomplete penetrance is demonstrated by the variable expressivity of the disease, mainly due to an interesting right-left and front-rear gradient, meaning that the right front foot is affected more often, followed by the left front (Fig. 16), right rear and left rear foot.

**Figure 16:** Holstein calf affected by Syndactyly; only the front legs show syndactylous hoof

## Inherited skin defect

*Ichthyosis* (64, 65, 66, 67, 68)

Ichthyosis is a rare skin disease reported in different breeds and characterized by diffuse cutaneous hyperkeratosis giving the skin an appearance similar to that of fish. Currently, two congenital forms of ichthyosis are described in cattle: ichthyosis fetalis and ichthyosis congenita.

Ichthyosis fetalis (bovine harlequin foetus) is the most severe form of bovine Icihthyosis and is incompatible with life; the affected calves are stillborn or die within a few days after birth. The skin is covered with large horny plates separated by deep fissures, resembling a 'leather cuirass'; hair is usually completely absent. The thick, inelastic skin causes eversion at mucocutaneous junctions, eclabium and ectropion. This form appears to be similar to human harlequin ichthyosis (HI) where there are severe plate-like cutaneous formations which are diffused over the body resulting in early neonatal death.

Ichthyosis congenita is the milder form of the disease; affected calves tend to live longer. General physical health is good. The lesions are characterized by hyperkeratosis, present at birth or developing over several weeks; hairlessness is not an initial feature, but alopecia may develop. Thick and large cutaneous scales are typical and more severe over the limbs, abdomen and muzzle

**Figure 17:** Three-month-old female Chianina calf affected by Ichtyosis congenita. Note the alopecic areas on the muzzle, eyelids and ears. The areas were covered by thick, dark-grey, dry, scale-like hyperkeratotic material. The diameter of the scales was 2-3 mm

(Fig. 17). Cataract, microtia and thyroid abnormalities have been reported in calves affected with this disorder. Bovine ichthyosis congenita resembles lamellar ichthyosis in humans (LI).

In both forms of ichthyosis, the constant histological feature, despite the variable anatomic sites and severity, is the marked orthokeratotic lamellar hyperkeratosis of the epidermis and follicular epidermis.

Presumably, as in the human forms of ichthyosis, the scales are the product of a defective desquamation associated with increased cohesion of keratinocytes and therefore represent a large number of cohesive corneocytes retained and shed simultaneously.

Both forms of ichthyosis in cattle are thought to be inherited through a simple autosomal recessive gene. By comparative analysis with the human forms of ichthyosis, a research project is underway at the University of Milan (Italy) where they are working on the candidate gene TGM1 (keratinocyte transglutaminase 1), responsible in man for the activity of TGase1, an enzyme in the cornified cell envelope assembly line.

Since neither chromosomal nor biochemical tests are yet available to detect carriers of this defect, reports of affected animals and the accurate identification of clinical cases are the only opportunity for carrier animals to be detected in retrospect.

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## DEDNE BOLEZNI PRI GOVEDU: IZBRAN PREGLED

A. Gentile, S. Testoni

**Povzetek:** V goveji populaciji je zaradi visoke stopnje medsebojnega parjenja nevarnost pojavljanja genetskih obolenj. Razširjena uporaba semena samo elitnih bikov poveča verjetnost pojavitve dveh mutiranih recesivnih genov v genotipu enega potomca.

Znaki, ki nakazujejo, da je bolezenska sprememba verjetno genetskega izvora, so:

1. bolezensko stanje je pogostejše v skupini sorodnih živali
2. bolezensko stanje opažamo v vseh letnih časih in na različnih geografskih področjih
3. stanje postaja pogostejše, ko se pogostnost medsebojnega parjenja povečuje

V zadnjem času smo imeli izkušnje predvsem z genetskimi obolenji.

Dedne bolezni centralnega živčnega sistema: *Mišična atrofija*, ki izvira iz hrbtenjače, je najbolj zaskrbljujoča dedna hiba sivorjavih telet, ki jo označuje huda mišična atrofija, progresivna pareza vseh okončin in ležanje na prsnici od tretjega ali četrtega tedna dalje. *Izguba mielina hrbtenjače* je prirojena nevrološka motnja, ki prizadene predvsem teleta sivorjave pasme. Ta po rojstvu ne morejo vstati in imajo tipično iztegnjene zadnje okončine. Prizadetost povzroča izguba mielina v belini hrbtenjače. *Goveja progresivna degenerativna mieloencefalopatija* (sindrom "tkalca") je dedna motnja čistokrvne sivorjave pasme. Zanjo je značilna progresivna šibkost zadnjih okončin in nekoordinirano gibanje, ki spominja na gibanje rok pri uporabi tkalskega čolnička (weaver syndrome - sindrom tkalca). Klinični znaki postanejo očitni med 6. in 8. mesecem starosti. *Spastična pareza* je najbolj problematična dedna hiba pasme romagnola. Napaka se odraža v hiperekstenziji skočnega sklepa in nenormalnem gibanju zadnje okončine. V najhujših primerih je prizadeta okončina stalno iztegnjena in nenehni krči povzročajo tipično gibanje v obliki nihala. Znaki spastične pareze se pojavijo med 3. in 8. mesecem starosti, ko spastični krči prizadenejo iztegovalke skočnega sklepa. *Spastični sindrom* je resen problem pri bikih v osemenjevalnih centrih, ki lahko prizadene tudi krave v progresivni ali ponavljajoči se obliki. Ponavljajoči se krči obojestransko prizadenejo notranje ledvene in križno-ledvene mišice. Pojavi se kifoza in drhtenje zadnjih okončin. Sčasoma se intenzivnost in trajanje krčev povečujeta.

Podedovane prirojene napake skeleta: *Hondrodisplazija* je znana tudi pod imenom "bulldog" telet in je bila včasih pogosta pri pasmi romagnola. Lobanja je sploščena, smrček kratek, čelo pa se le počasi vzdiguje. Prizadete živali imajo tudi krajše okončine. Pri t. i. *"prirojenem sindromu telečjega vampa"* pasme romagnola poleg skrajšane in sploščene lobanje

opazimo še volčje žrelo in napihnjenost trebuha zaradi znatno povečane količine tekočine v trebušni votlini. *Kompleksna nepravilnost vretenc* (CVM - complex vertebral malformations) predstavlja najresnejšo dedno napako telet črno-bele pasme. Teleta so manjša, imajo simetrično artrogripozo in popačeno obliko vratnih ali prsnih vretenc. Najpogosteje opazimo skrajšan vrat in skoliozo. Anomalije na vretencih so lahko hemivretenca, združevanje vretenc med sabo in vretenc z rebri, skrivljenost reber in nepravilne oblike na vretencih. Homozigoti pogosto poginejo že pred rojstvom. *Arahnomelia* je prirojena napaka skeletnega sistema, ki daje novorojeni živali pajkast videz. Opisana je pri teletih sivorjave pasme in simentalcih. Deformirane so predvsem obrazne kosti in distalni deli zadnjih okončin, opisujemo tudi kostno dolihostenomelijo (nenormalno dolge in tanke okončine). Kosti okončin so tudi bolj krhke in spontani zlomi med telitvijo lahko poškodujejo kravo. *Smrtonosno skrajšanje hrbtenice* je redka napaka skeletnega sistema. Teleta so bistveno manjša in hrbtenica je nesorazmerna. Vretenca so lahko združena, njihovo število je različno, samo telo vretenc pa je nepravilne oblike in velikosti. V vratnem in prsno-ledvenem področju vretenca pogosto tudi niso poravnana. *Perosomus acaudatus* in *Perosomus elumbis* sta izraza, ki označujeta nepopolno razvitost hrbtenice. Teletu lahko manjka le rep (Perosomus acaudatus) ali pa tudi del križnega, celo ledvenega dela (Perosomus elumbis). Manjkajoči rep po navadi ne predstavlja nevarnosti za življenje, medtem ko teleta s Perosomus elumbis ne preživijo, saj manjka čvrsta opora za zadnje okončine, ki so pri tem stanju tudi zelo nerazvite. *Sindaktilija* ("noga mule") je znana napaka, pri kateri so prsti združeni. Izraženost napake zelo variira. Običajno so bolj prizadete sprednje okončine in desna stran bolj od leve.
Prirojene napake kože: *Ihtioza* je difuzna hiperkeratinizacija kože. Koža je podobna ribji, od tod ime. Poznamo dve obliki ihtioze. *Ichthyosis fetalis* ali "goveji harlekin fetus" je prirojena in zelo resna napaka, ki ni združljiva z življenjem. *Ichthyosis congenita* je milejša oblika in prizadete živali lahko živijo dlje. V obeh primerih je koža pokrita s velikimi bodičastimi ploskvami, med katerimi so globoke zareze, kot nekakšen usnjen oklep.

**Ključne besede:** dedne bolezni; centralni živčni sistem, bolezni – genetika; kožne bolezni – genetika; kost, bolezni – genetika; govedo

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**Review Paper**

# HIGH MILK PRODUCTION AND GOOD FERTILITY IN MODERN DAIRY COWS: THE RESULTS OF SOME RECENT RESEARCH ITEMS

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**Summary:** The present manuscript summarizes the research, which is currently going on in our department regarding the interaction between negative energy balance (NEB) and fertility in modern high yielding dairy cows. In this work, it has been clearly demonstrated that nearly 50% of the recently calved dairy cows suffer from one or another ovarian dysfunction during the preservice postpartum period. Both clinical signs of a significant negative energy balance as well as the occurrence of puerperal disorders were main risk factors to suffer from these ovarian problems.
In the following research using repeated ovum pick sessions it was demonstrated that homeorhetic changes of metabolites known as typical indicators of the negative energy balance, were reflected in the follicular fluid of the dominant follicle during the immediate postpartum period. Based on *in vitro* experiments it was furthermore shown that these metabolites were able to affect bovine granulosa cells at concentrations, which were found in the in vivo study. The latter opens perspectives in elucidating the question why high yielding dairy cows express less heat symptoms and why modern dairy cows are at an increased risk to suffer from postpartal ovarian dysfunctions such as cystic ovarian disease.
Besides the effect on granulosa cells, studies were also carried out to investigate the effect of elevated levels of non esterified fatty acids (NEFAs) on the reproductive competence of the oocyte. In these studies it was demonstrated that NEFAs at levels, which could be found *in vivo* within the follicular fluid of the dominant follicle during NEB, may influence fertility of high yielding dairy cows by hampering the oocyte maturation as expressed in lower fertilization rates and subsequently lower cleavage and blastocyst development. The latter opens possibilities to explain the worldwide mentioned decreasing fertility results seen in modern high yielding dairy cows

**Key words:** Cattle diseases - etiology; fertility; energy metabolism; follicular fluid - chemistry; fatty acid, nonestrified - analysis; ovarian follicle; postpartum period; cattle - female

## Introduction

Currently farmers, veterinarians, consultants and even researchers are very demanding for our dairy cows. Besides the demand to produce lots of milk containing high levels of protein, we want them to calve each year. The latter implies that we like every dairy cow to be pregnant or to be recovering from pregnancy and preparing for a new pregnancy at all times, which makes fertility a full time job for her. Getting pregnant during the first 85 days after calving demands, however, a supreme co-operation of the involuting uterus, the hypothalamus, the pituitary, and the ovaries, leading to an undisturbed involution of the uterus, to the resumption of normal ovarian cyclicity, to the expression of heat symptoms and finally to conception. It is clear that the most important events concerning reproduction occur while the cow is at its peak production and experiences severe metabolic stress. At the Ghent University research is going on to reveal the effects of a high level of production on the fertility of the modern dairy cow, and to detect the underlying causes of fertility disturbances. The current review summarizes some of the results of this research.

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## Decreasing fertility?

Many studies report a decrease in the fertility of modern dairy cows. Since these studies originate from regions all over the globe, the situation seems to be widespread and universally accepted. In the United States, for example, the conception rate has been reported to decrease by 0,45% per year over a twenty year period (1). In the UK this decrease has been in the order of 1% per year (2,3). As comparable results from several other countries continue to appear, these reports have provoked an alarm response that goes on unabated. While over the same time period, significant increases in the level of milk production were reported, people got tempted to blame the increase in milk production to cause the decrease in fertility. In Flanders, average milk production of Holstein Fresian cows increased from 7.496 liters in 1995 towards 8.440 liters in 2000. During the same time period, the calving interval increased from 399 towards 407 days, while the 56-day non-return rate remained relatively stable (69,7 in 1995 and 69,9 in 2000). Analyses of fertility data of the local AI center revealed that the prolongation of the calving interval was mainly due to a prolongation of the interval from parturition to first insemination, while the data expressing the ability of the cows to conceive (i.e. pregnancy rate after first and second insemination, number of inseminations per pregnancy) were not associated with the prolongation of the interval between two consecutive calvings. Hence, we came to the conclusion that at least in Flanders the decrease in fertility of dairy cows as expressed by a prolongation of the calving interval, was mainly due to the inability of the farmers to see their cows in heat at the moment they should inseminate them.

*Anoestrus main problem*

Based on the aforementioned knowledge, more research was set up to further elucidate the anoestrus problem in Flemish high-yielding dairy herds. In a detailed study examining fertility data of 3.108 lactations (4), in 1.291 (42%) of all studied lactations no heat was observed within 60 days after calving. Cows not seen in heat within 60 days after calving had an average increase in days open with 26 days (days open: 111 vs. 85 days). Of the 1.817 cows which had been seen in heat during the first 60 days after calving, 622 (34%) had to be examined later on because they had not been seen in heat at the time they should be inseminated. The latter cows were described as suffering from 'a cessation of observed heat symptoms', and had an average increase of 24 days in the interval from calving to conception (days open: 109 vs. 85). Hence in total, 1.913 (62%) off all lactations were identified as having suffered from one or another kind of preservice postpartum anoestrus. Both cows not seen in heat within 60 days after calving and cows suffering from 'cessation of observed heat symptoms' had a significantly increased risk of being culled in the current lactation.

*Abnormal ovarian activity post partum*

The aforementioned results led to the question whether the anoestrus problem is merely due to shortcomings in the management (e.g. failure to detect estrus) or whether it is peculiar to the modern high-yielding dairy cow herself. Furthermore, when problems could indeed be designated as being inherent to the high-yielding dairy cow, the next question arises as whether the anoestrus problems are caused by a lack of expressing heat symptoms by the cow, or by ovarian/uterine disorders leading to the symptom of anoestrus. In order to investigate this into more detail, further research was carried out based on the analysis of milk progesterone profiles (5). Although it is nearly impossible to compare the results of different studies because of different sampling protocols and the use of different definitions for both normal and abnormal progesterone profiles, authors nowadays come to very comparable conclusions. The first significant rise in progesterone is stated to occur on the average at 37 days after calving (5), indicating that the first postpartum ovulation in the modern-day dairy cow occurs around day 30 after calving. The very wide range and standard deviation mentioned, however, suggested the presence of many cows with ovarian abnormalities. The latter was confirmed by the same study, in which 47% of the 448 examined progesterone profiles showed an abnormal pattern during the preservice postpartum period. The two most frequently recognized abnormalities were delayed cyclicity or anovulation (= no significant progesterone rise during the first 50 days after calving), and prolonged luteal phase (= a period of at least 20 days of positive progesterone levels without a preceding insemination). In comparison with moderate yielding Friesians, modern high yielding Holsteins showed an increased incidence of postpartum abnormal ovarian cycles (Table 1).

By means of regular rectal palpations, we

found that small, inactive ovaries and not cysts were the most important reason of delayed cyclicity. Searching for the causes of prolonged luteal phases, in almost half (48%) of these cows an abnormal uterine content could be palpated, in 3% a cyst-like structure on one of the ovaries was discernable while on 49% no specific reasons for this ovarian abnormality could be found (5).

**Table 1:** A comparison of postpartum reproduction parameters based on measurement of progesterone in milk twice weekly in two different studies using moderate yielding Friesians (6) or modern high yielding Holsteins (5)

| Ovarian activity based on progesterone profiles | Traditional herds (Fagan and Roche, 1986) | Modern high yielding dairy herds (Opsomer et al., 1998) |
|---|---|---|
| Number of cycles | 448 | 448 |
| Number of cyclical patterns (%) | 78 | 53 |
| Delayed cyclicity (%) | 7 | 20,5 |
| Temp. cessation of cyclicity (%) | 3 | 4 |
| Prolonged luteal phase (%) | 3 | 20,5 |
| Short cycles (%) | 4 | 0,5 |
| Other irregular patterns (%) | 4 | 1,5 |

Based on a multivariate analysis at farm level, taking into account a number of relevant factors, we demonstrated that calving during the stable period, an extended length of the previous dry period, health problems during the first month of lactation and clinical parameters illustrating the appearance of a severe negative energy balance (NEB), significantly increase the risk for delayed cyclicity before service. Parity, problem calvings, health problems during the first month of lactation and (too) early resumption of ovarian cyclicity after calving significantly increase the risk for prolonged luteal cycles before service (7). Hence, these field studies clearly confirmed previously described clinical trials, in which the health status and the NEB of the animals shortly after calving were demonstrated to be the most important risk factors leading to delayed cyclicity and anovulation, while the occurrence of prolonged luteal cycles is not directly dependent on the energy balance of the animals, but is mainly caused by puerperal disturbances. The latter is furthermore enhanced by the fact that cows in NEB do suffer from a reduced immunity by means of a decreased killing activity of the neutrophils, which renders them more susceptible towards different kinds of puerperal infections (8).

*Causal relationship between negative energy balance and fertility problems*

Although it is nowadays generally accepted that the NEB during early lactation is associated with many ovarian disturbances, there remain a lot of questions about the possible mechanisms lying behind this association. Hence, much research is currently going on to investigate further the relation between NEB and fertility in the dairy cow. As the typical homeorhetic changes of several hormones and metabolites are known to act as specific markers for the adaptation of the cows to the metabolic challenge they face during the first weeks after calving, investigations have been done to see whether elevated or lowered levels of these metabolites may be seen as the link between the NEB and the fertility decrease we currently notice in the modern-day dairy cow. As elevated serum concentrations of non esterified fatty acids (NEFAs) are an important characteristic of the cow in NEB, NEFAs have been tested to see whether they may have a negative impact on fertility. As studies until now failed to show a clear relationship between LH pulsatility characteristics, and the concentrations of several energy metabolites in the immediate postpartum period (9), researchers started to focus more and more on the effect of these metabolites at the ovarian level. In vivo, increased NEFA concentrations are correlated with lowered progesterone concentrations and a decrease in the weight of the corpus luteum (CL). Furthermore, other studies found lower CL weights in animals during NEB. Hence, while it is currently accepted that elevated NEFA concentrations are indeed good indicators of the NEB in the recently calved dairy cow, and while there are serious indications that those elevated NEFA levels have a detrimental effect on the ovary, it is not known to which degree the plasma NEFA concentrations are reflected in the follicular fluid at different time points after calving and what kind of effect these elevated NEFA concentrations may have on the different cell types of the ovary.

*Recent research*

Currently, at our department research is going on to investigate to what extent metabolic changes that occur in early postpartum high-yielding dairy cows are reflected in the follicular fluid (FF) of the dominant follicle (>8mm) (10). Nine blood samples were taken per cow from nine high-yielding dairy cows between 7 days before and 46 days after parturition. From day 14 post partum on and together with blood sampling, FF samples of the largest follicle were collected from the same cows by means of transvaginal follicle aspiration. Serum and FF samples were analyzed using commercial clinical and photometric chemistry assays for glucose, β-OH butyrate (β-OHB), urea, total protein (TP), triglycerids (TG), NEFA and total cholesterol (TC). All cows lost body condition during the experimental period, illustrating a NEB during the experimental period. In FF, glucose concentrations were significantly higher and the TP, TG, NEFA and TC concentrations were significantly lower than in serum. The concentrations of glucose, β-OHB, urea and TC in serum and in FF changed significantly over time (P<0.05). Throughout the study, changes of all metabolites in serum were reflected by similar changes in FF (Figures 1-3). Especially for glucose, β-OHB and urea, the correlations were remarkably high. The results of that study confirm that the typical metabolic adaptations which can be found in serum of high-yielding dairy cows shortly post partum, are reflected in FF (Table 1) and, therefore, may affect the quality of both the oocyte and the granulosa cells (10).

Based on the study of Leroy et al. (10), we knew the concentration of several metabolites like NEFA in the FF. In the next study (11) we tested the effect of the most abundant NEFA (oleic- (OA), C18:1; stearic-(SA) C18:0 and palmitic acid (PA) C16:0) on granulosa cell proliferation using the concentrations which were measured *in vivo* by Leroy et al. (10). Granulosa cells were harvested through repeated aspiration of follicular fluid from large follicles (>8mm) on slaughterhouse ovaries. Cells were cultured for 48h under serum free conditions with 1 ng/ml FSH and 10 ng/ml insulin. Cells were treated with 0, 150, 300 or 500 μM of the individual fatty acid or 450 μM of a 1:1:1 combination of all three fatty acids. At the end of the culture, granulosa cell numbers were determined spectrophotometrically. Both PA and SA had a significant inhibitory effect on granulosa cell proliferation at the three concentrations tested (P<0,01). This effect was not dose dependent for PA (P>0,05) since all three concentrations reduced cell numbers evenly (52,9 to 60% reduction). Stearic acid, on the other hand, had a more severe negative effect on cell proliferation at 300 μM and 500 μM than at 150 μM (P<0,01). Oleic acid only inhibited cell proliferation significantly (P<0,01) at the highest concentration of 500 μM (66,5% reduction). The combination treatment also reduced cell numbers significantly (P<001) in comparison to controls (34,7% reduction). It could be concluded that *in vitro,* NEFAs reduce cell proliferation and/or survival of bovine granulosa cells. The latter indicates that elevated NEFA concentrations may affect ovarian cells and hence ovarian functioning contributing to the decrease in fertility, which is currently mentioned in high yielding dairy cows (11).

In a more recent study, we furthermore investigated the effect of elevated NEFA levels on the oocyte quality, testing their effect on fertilisation rate, cleavage and subsequent blastocyst formation using an *in vitro* model (12) (Tables 3 and 4).

It was concluded that high levels of NEFAs during a period of NEB might influence fertility of high yielding dairy cows by hampering the oocyte maturation as expressed in lower fertilization rates and subsequent lower cleavage and blastocyst development. Using a new lipid analysis technique to evaluate the lipid content of single bovine oocytes and embryos we were furthermore able to demonstrate a significant increase of the lipid content of in vitro produced embryos, after culture in the presence of serum (13).

**Table 2:** Correlation coefficients (r's) between metabolite concentrations in follicular fluid and serum per experimental session in nine dairy cows

| Correlations (*r*) | Glucose | β-OHB | Urea | Total Protein | Triglycerides | NEFA | Total Cholesterol |
|---|---|---|---|---|---|---|---|
| 14 days pp | 0.834* | 0.996** | NS | NS | 0.892** | NS | NS |
| 20 days pp | 0.788* | 0.972** | 0.929** | NS | NS | NS | 0.787* |
| 26 days pp | 0.733* | 0.992** | 0.987** | NS | 0.872** | NS | NS |
| 33 days pp | 0.925** | 0.976** | 0.990** | NS | 0.710* | 0.845** | 0.918** |
| 40 days pp | 0.916** | 0.971** | 0.973** | 0.860** | NS | NS | 0.862** |
| 46 days pp | 0.901* | 1.00** | 0.782* | NS | NS | 0.908* | 0.948* |

Values are presented for significant correlations (* $P < 0.05$; ** $P < 0.01$; NS: not significant).

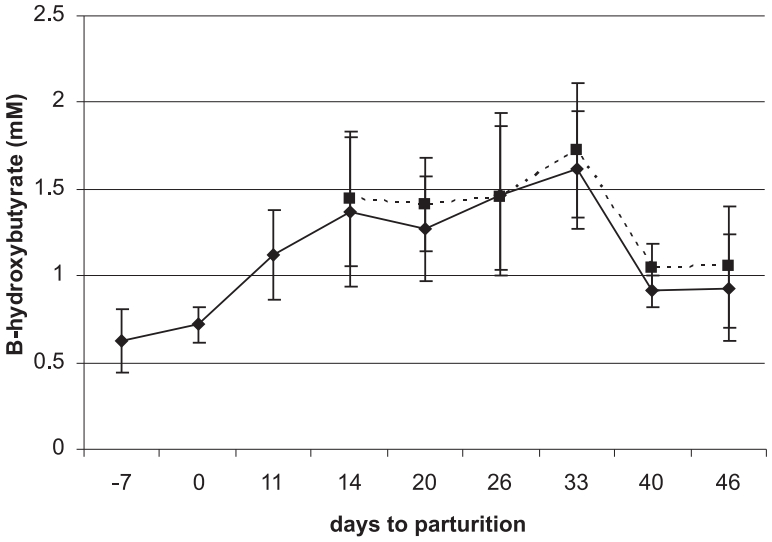


**Figure 1:** Average (± SEM) β-hydroxybutyrate concentrations (mM) in serum (full line) and follicular fluid (dotted line) of 9 high yielding dairy cows during the experimental period

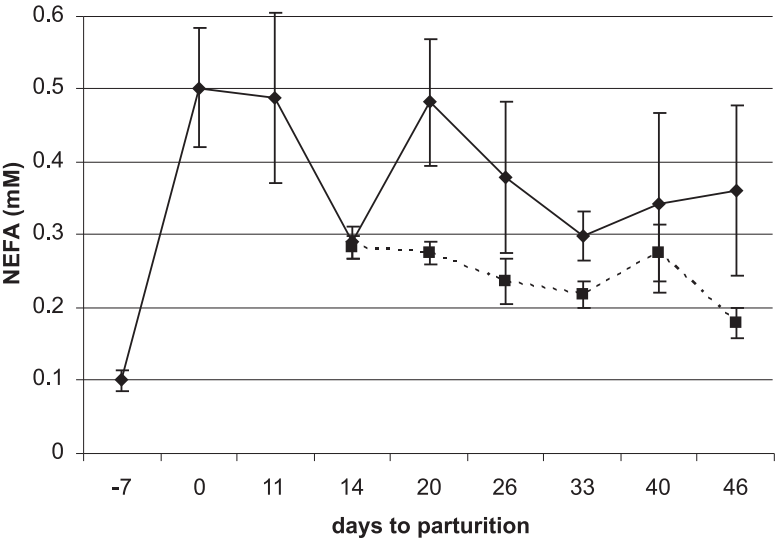


**Figure 2:** Average (± SEM) Nefa concentrations (mM) in serum (full line) and follicular fluid (dotted line) of 9 high yielding dairy cows during the experimental period

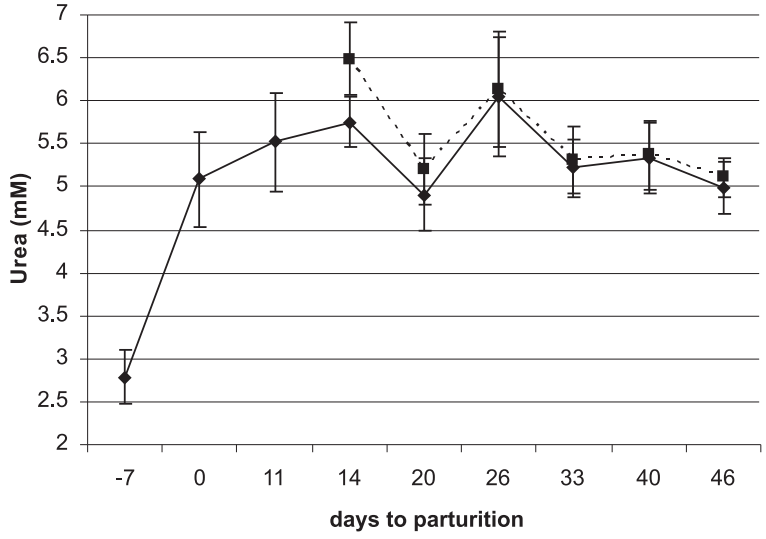


**Figure 3:** Average (± SEM) Urea concentrations (mM) in serum (full line) and follicular fluid (dotted line) of 9 high yielding dairy cows during the experimental period

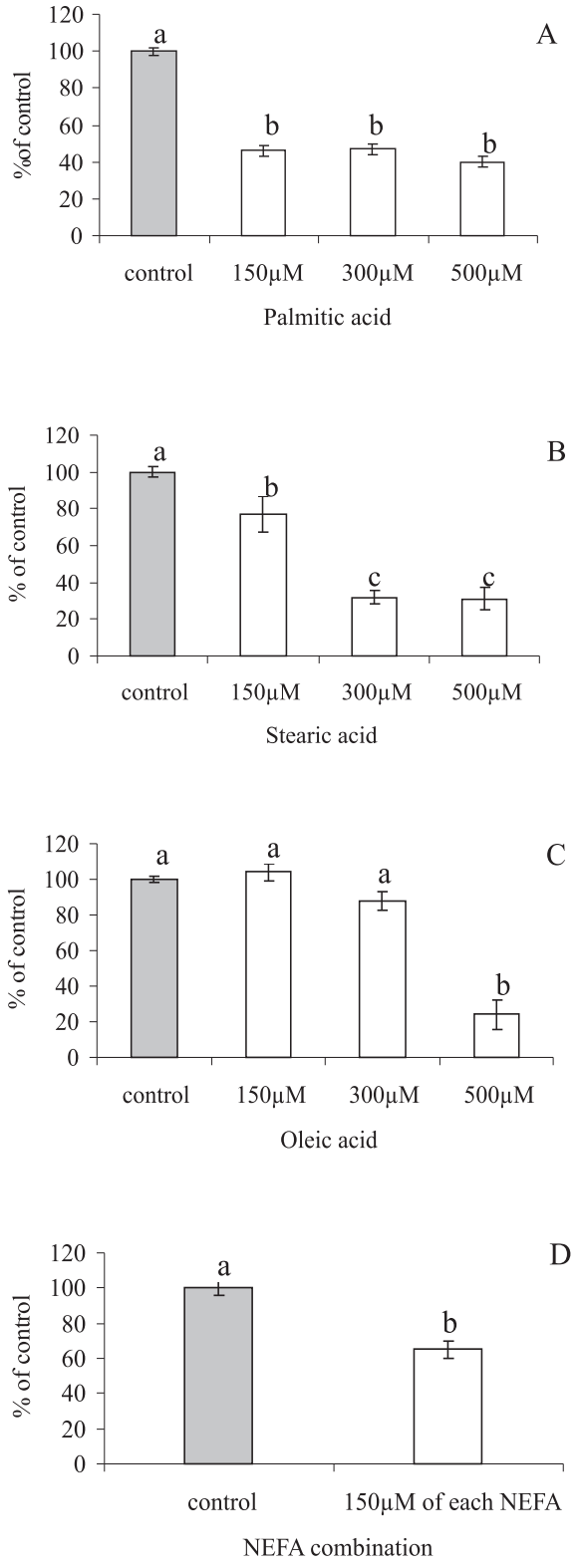


**Figure 4:** Effects of different concentrations of palmitic (A), stearic (B) and oleic (C) acid alone or combined (D) on granulosa cell proliferation (cells per well; mean – SEM) after 48 h of culture. The NEFA-combination (D) contains 150µM of each of the fatty acids. Data are expressed as percentage of controls. Means with diffe-rent superscripts differ significantly ($P<0.05$)

**Table 3:** Effect of stearic acid (C18:0) added to the maturation medium on maturation and fertilization rate, cleavage rate (± SEM) at 48h after fertilization (pi) and number of blastocysts (± SEM) at 8 days pi relative to the number of bovine oocytes put in culture or relative to the cleaved zygotes

| | Negative control | Positive control | Stearic acid (C18:0) |
|---|---|---|---|
| Maturation rate (%) | | | |
| Metaphase I | 9.2[a] | 18.6[b*] | 26.0[b*] |
| Ana-/Telophase[1] | 16.1[a] | 11.6[a] | 18.4[a] |
| Metaphase II | 74.8[a] | 67.8[a] | 54.0[b] |
| Fertilization rate (%) | | | |
| Metaphase II | 10.7[a] | 8.8[a] | 23.4[b] |
| 2 Pronuclei | 69.7[a] | 72.2[a] | 55.6[b] |
| > 2 Pronuclei | 12.5[a] | 12.1[a] | 12.5[a] |
| Cleavage rate at 48h pi (%) | 76.9 ± 3.2[a] | 77.4 ± 2.7[a] | 57.9 ± 3.6[b] |
| % blastocysts from oocytes | 33.3 ± 3.6[a] | 34.4 ± 2.1[a] | 21.3 ± 3.5[b] |
| % blastocysts from cleaved | 43.1 ± 4.3[a] | 44.4 ± 2.1[a] | 39.6 ± 7.0[a] |

[a,b] Data within a row marked with different superscripts, differ significantly (P < 0.05).
[*] *P* = 0.1
[1] Significant interaction term "treatment X replicate".

**Table 4:** Effect of palmitic acid (C16:0) added to the maturation medium on maturation and fertilization rate, cleavage rate (± SEM) at 48h after fertilization (pi) and number of blastocysts (± SEM) at 8 days pi relative to the number of bovine oocytes put in culture or relative to the cleaved zygotes

| | Negative control | Positive control | Palmitic acid (C16:0) |
|---|---|---|---|
| Maturation rate (%) | | | |
| Metaphase I | 9.1[a] | 12.5[a] | 24.1[b] |
| Ana-/Telophase | 15.9[a,b] | 10.5[a] | 19.9[b] |
| Metaphase II | 75.0[a] | 77.1[a] | 63.2[b] |
| Fertilization rate (%) | | | |
| Metaphase II | 21.6[a] | 20.2[a] | 33.5[b] |
| 2 Pronuclei | 64.0[a] | 59.2[a] | 43.4[b] |
| > 2 Pronuclei[1] | 7.0[a] | 5.8[a] | 11.6[a] |
| Cleavage rate at 48h pi (%) | 76.6 ± 2.3[a] | 74.5 ± 2.6[a,b*] | 66.6 ± 3.2[b*] |
| % blastocysts from oocytes | 22.4 ± 2.0[a] | 24.6 ± 1.5[a$] | 17.2 ± 3.0[a$] |
| % blastocysts from cleaved | 29.1 ± 2.4[ab§] | 33.2 ± 1.8[a] | 22.7 ± 4.1[b§] |

[a,b] Data within a row marked with different superscripts, differ significantly (P < 0.05).
[1] Significant interaction term "treatment X replicate".
[*] *P* = 0.07
[$] *P* = 0.06
[§] *P* = 0.12

*How to translate this knowledge towards practice?*

The biggest challenge for practitioners is to 'translate' this knowledge into practice and use it to help the herds they have in their herd health control program to reach an acceptable level of reproduction. As modern herd health control programs should focus on taking preventive measures rather than on increasing curative treatments (14), not only modern cows but also their 'coaches' have to adapt to the current levels of milk production. This adaptation has to do with an optimalization of the management! While reproduction is a full time job for the dairy cow, coaching her to reproduce well takes no less time.

Based on the above it is clear that implementing a dairy herd fertility control program should definitely be more than putting our arms in cows' rectums to examine cows with problems. Giving advice upon the management of the dairy 'top athletes' to prevent fertility problems for sure needs at least the same amount of energy. The challenge is to integrate the current knowledge into nutritional management, production medicine, and reproductive management procedures taking into account the specific obstacles each individual herd has to face, to finally optimize fertility of the herd (15). In the absence of such a holistic approach, the response to traditional veterinary therapies and herd health programmes may become increasingly diminished.

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## VISOKA PROIZVODNOST IN DOBRA REPRODUKTIVNA SPOSOBNOST PRI SODOBNIH KRAVAH MOLZNICAH: REZULTATI NEKATERIH NOVIH RAZISKAV

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**Povzetek:** V članku je predstavljen pregled raziskav, ki jih trenutno izvajamo na našem oddelku glede medsebojnih odnosov med negativno energetsko bilanco (NEB - negative energy balance) in reprodukcijsko sposobnostjo pri novejših pasmah visokoproizvodnih krav molznic. Z raziskavami smo nedvomno dokazali, da ima skoraj 50 % krav, ki so pred kratkim telile, okvarjeno delovanje jajčnikov med predservisnim poporodnim obdobjem. Težave z jajčniki nastanejo tako zaradi izrazite negativne energetske bilance kot zaradi poporodnih motenj.
V raziskavi smo pri kravah iskali zoreče jajčne celice in ugotovili, da se homeoretične spremembe presnovkov, ki so sicer značilni pokazatelji NEB, odražajo tudi v folikularni tekočini dominantnega folikla v zgodnjem poporodnem obdobju. Poskusi in vitro so nadalje pokazali, da so ti presnovki v koncentracijah, kot smo jih določili in vivo, vplivali na granulozne celice jajčnikov. To odkritje nudi nove možnosti razjasnjevanja, zakaj imajo visokoproizvodne krave molznice slabe znake pojatve in zakaj je pri novejših pasmah molznic visoka verjetnost poporodnih motenj, kot je npr. cistično obolenje jajčnikov. Ker smo ugotovili učinek na granulozne celice, smo preiskali tudi učinek povišanih vrednosti neestrificiranih maščobnih kislin (NEFA) na reprodukcijsko sposobnost jajčne celice. Ugotovili smo, da imajo lahko NEFA v koncentracijah, kot jih najdemo in vivo v folikularni tekočini dominantnega folikla, negativen vpliv na reprodukcijo visokoproizvodnih krav. Zavirajo namreč zorenje jajčne celice, kar se odraža v nižji stopnji oploditve in slabšem razvoju blastociste. To dejstvo lahko razloži slabše reprodukcijske rezultate, ki jih opažamo pri novejših pasmah krav molznic po celem svetu.

**Ključne besede:** govedo, bolezni - etiologija; plodnost; energetski metabolizem; folikularna tekočina - kemija; maščobne kisline neestrificirane - analize; ovarijski folikel; poporodno obdobje; krave

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**Review article**

# *STAPHYLOCOCCUS AUREUS* - DO WE REALLY HAVE TO LIVE WITH IT?

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**Summary:** Staphylococcus aureus (S. aureus) is still one of the most prevalent mastitis pathogens in dairy herds all over the world. Effective and economic S. aureus control programs rely on prevention rather than treatment. Since the introduction of the standard mastitis prevention program, much progress has been achieved in decreasing the prevalence of intramammary infections (IMI). However, at the farm level, staphylococcal mastitis remains the disease causing the highest financial losses. Among S. aureus strains isolated from the bovine mammary gland resistance to penicillin increased rapidly from approximately 20 % in 1965 to 45 % in the mid 70s and decreased again in the 1990s to approximately 30 %. Although the therapeutic value of penicillin is limited in many countries, there are still sufficient antimicrobials available for treatment of S. aureus IMI. Currently there are no founded indications that methicillin-resistant S. aureus (MRSA) strains are involved in bovine mastitis. To control S. aureus mastitis at the farm level complex measurements, which involves strategies for treatment of existing infections and also prevention of new mastitis cases should be implemented.

**Key words:** cattle diseases – preventive and control; mastitis, bovine – drug therapy; *Staphylococcus aureus*; cattle – female

## Introduction

Staphylococci are Gram-positive spherical bacteria that occur in microscopic clusters resembling grapes. In 1884, Rosenbach described two pigmented colony types and proposed the appropriate nomenclature: *Staphylococcus aureus (S. aureus)* (yellow) and *S. albus* (white). The latter species is nowadays named *S. epidermidis* (1). Until now more than 40 different species and many subspecies in the genus *Staphylococcus* have been described. Staphylococci are found worldwide in warm-blooded animals.

Among bacterial species *S. aureus* is one of the most frequently isolated major bovine mastitis pathogen (2).

Mastitis is the most common and costly production disease affecting dairy cows. Many interpretations of the word “mastitis” exist in research and in farming practice. Literally, “mastitis” means “inflammation of mammary gland tissue”. Inflammation of the bovine udder is usually caused by infection, mostly by bacteria, yeasts or fungi, but it can also be the result of sterile inflammation due to chemical, physical or mechanical trauma (3).

In spite of many proofs for contagious character of *S. aureus,* the bacterium is ubiquitous on dairy farms. *S. aureus* strains can be isolated from healthy bovine teat skin, human skin, milking equipment and bovine milk (4).

Although *S. aureus* can, usually, be effectively combated with the 5-point program, later extended to the 10-point program, including segregation, it still causes problems on dairy farms, making clear that it is difficult to control *S. aureus* mastitis and that it may be impossible to eradicate the disease (5). Most herds do not have facilities or labour to handle additional groups or individual mastitic animals and are not willing to cull infected animals. Therefore, in recent years more emphasis has been placed on the treatment rather than prevention. However, little progress had been made during the previous ten years towards solving some of the basic problems associated with antimicrobial treatment of staphylococcal mastitis, i.e. the low cure rate for clinical and subclinical *S. aureus* infections. Staphylo-

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cocci spread by direct or indirect contact, but interspecies spread (e.g. humans - cows, dogs - humans) appears to be limited (6). Many animal infections are probably endogenous, that is, caused by a resident strain. The objectives of the article were to review current knowledge about *S. aureus* epidemiology, pathogenesis, diagnosis, treatment and control.

## Epidemiology and importance of S. aureus mastitis

The most common transmission pathway occur through transfer from an infected mammary gland to an uninfected gland via devices, such as milking equipment, common udder cloths, or the milker's hands. In herds that do not practice backflushing, residual milk remains in the teat cups. If the last cow milked with that unit had a *S. aureus* udder infection, then the next cow, milked with the same unit, will be directly exposed to the pathogen. If employed, common clothes or sponges can be a major means of spreading *S. aureus* as nearly every cow in the herd would be exposed on a daily basis. The importance of the milker's hands in spreading *S. aureus* could be equally as important as a common udder cloth, especially in herds that practice forestripping. The milking parlor and the lactating period represent the place and time period where most new IMI occur, but *S. aureus* can result in new IMI during the dry period and also in heifers. Although the infected mammary gland could still be the source of these infections, it is obviously not the only reservoir of *S. aureus* on dairy farms (7).

In many countries the number of mastitis cases, particularly subclinical, caused by *S. aureus* is still very high. In Slovenia, for example, the proportion of *S. aureus* udder infections was 48,2 % in 1997, 45 % in 1998, 48 % in 1999 and 53,7 % in 2000 (8). Comparable results were found in the Netherlands 17,2 % in 1950, 42,8 % in 1975 and 40,5 % in 2000 (9). In Italy the prevalence of subclinical *S. aureus* udder infections was 21 % in 2001 (10). In an Austrian study from 2003, *S. aureus* was found in 43 % of subclinical mastitis cases (11).

## Pathogenesis

*S. aureus* expresses many potential virulence factors:

- surface proteins that promote colonization of host tissue
- invasins that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase)
- surface factors that inhibit phagocytic engulfment (capsule, protein A)
- biochemical properties that enhance their survival in phagocytes (carotenoids, catalase production)
- immunological disguises (Protein A, coagulase)
- membrane-damaging toxins that lyses eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin)
- exotoxins that damage host tissues or otherwise provoke symptoms of disease
- inherent and acquired resistance to antimicrobial agents (12)

For the majority of diseases caused by *S. aureus*, pathogenesis is multifactorial, so it is difficult to determine precisely the role of any given factor. However, there are correlations between strains isolated from particular diseases and expression of particular virulence determinants, which suggests their role in a particular disease. In the last decade the application of molecular biology has led to advances in unraveling the pathogenesis of staphylococcal diseases. Genes encoding potential virulence factors have been cloned and sequenced, and many protein toxins have been purified (13).

Infection of the mammary gland can start when *S. aureus* cells penetrate the teat canal. Most bacteria penetrate the teat during milking, when the sphincter muscle is relaxed. Shortly after penetration, the bacteria rapidly multiply in milk and adhere to epithelial cells. Bacteria that have not adhered to epithelial cells will be removed during next milking. In case of successful colonization, large quantities of bacteria can be found in milk after twenty-four hours. Four days after infection, *S. aureus* is already present in interstitial tissue and also intracellularly in epithelial cells. In the beginning of staphylococcal udder infection, only small areas of the gland may be involved. Cells of the alveoli and ducts gradually degenerate and slough from the cistern lining and, together with somatic cells, occlude milk ducts that drain milk-producing areas. This obstruction leads to involution of the remaining functional alveoli and formation of scar tissue. Occluded ducts may reopen, releasing pathogens to other areas of the gland. This process is then repeated, initiating a continuous cycle of infection of different areas of the quarter. During the early stages of infection, tissue damage is minimal and reversible. If effectively treated, the quarter will return to near normal milk production in subsequent lactations. If microorganism remains within the occluded area, abscesses may become quite large and can be palpated in the udder tissue (14).

## Symptoms

In many countries *S. aureus* is the most common cause of subclinical udder infection but not necessarily of clinical mastitis. It does not often cause peracute mastitis, usually producing a chronic disease with occasional occurrences of clinical mastitis. Partly due to the poor response to antibiotic therapy it is the most persistent of infections, frequently lasting for several months and even years (15). In cows strains of *S. aureus* rarely produce toxins that cause blood vessel constriction and massive clotting, which leads to interruptions in the blood supply of the affected area. The consequence is a gangrenous course of mastitis, which is uncommon in the bovine mammary gland, but very frequent in small ruminants (16).

## Diagnosis

*S. aureus* is a Gram-positive, facultative anaerobic, non-sporeforming coccus, belonging to the family of *Micrococcaceae*. The bacterium is coagulase and catalase positive and oxidase negative. In the laboratory beside the Gram stain, the catalase and the slide coagulase test are performed to differentiate *S. aureus* from other species.

*S. aureus* forms on blood agar large colonies with characteristic pigmentation and hemolytic patterns (17). To identify staphylococcal isolates at the species level, commercial biochemical tests are available (18).

## Treatment and antimicrobial susceptibility

Susceptibility testing is by far the most important laboratory test used in selection of the therapeutically relevant antimicrobial drug. The clinicians rely increasingly on this test, as it is the only relevant information applied for therapy design. However, these tests have numerous shortcomings concerning their technical performance as well as the interpretation of the results. Even if the susceptibility testing was correctly performed and interpreted, very little is understood about the implications concerning selection of specific drug formulations, adequate dosage and sufficient dosing interval. The test result that the clinician receives from the laboratory, relate to the established break point values. These values are largely unknown to the clinician. Therefore, the clinician is unaware of what the target plasma concentrations should be and how they can be reached. However, this particular information is critical for selecting the correct therapeutic agent. Moreover, it is crucial to select the right pharmaceutical formulation of the appropriate drug. For instance, equal doses of benzylpenicillin G, procain penicillin and benzathine penicillin will produce completely different penicillin plasma concentrations (19). Consequently, the results of in vitro susceptibility testing can provide only restricted therapy guidelines.

The development of bacterial resistance has nearly always followed the therapeutic use of antimicrobial agents. When penicillin was introduced for clinical use in 1941, virtually all strains of *S. aureus* were susceptible. In 1944, first reports on penicillin resistant *S. aureus* strains appeared, and within less than a decade, serious resistance problems have been observed in many countries (20). 1960 new β-lactamase-resistant antibiotics (methicillin) were developed to fight staphylococcal infections, but 15 years later the first methicillin-resistant *S. aureus* (MRSA) strains emerged. Afterwards vancomycin was the drug of choice to treat these infections. Finally, in 1996 vancomycin-resistant (VRSA) strains were reported from Japan. Nowadays, *S. aureus* is consistently one of the top four causes of nosocomial infections in humans, along with *Escherichia coli*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* (21).

In cattle the rate of narrow-spectrum penicillin resistance *S. aureus* strains varies per country and also over time within countries. For example, in the U.K., penicillin-resistance in *S. aureus* isolated from bovine mastitis has increased from 2 % in 1949 to approximately 70 % in the 1980s (22). However, most recent results indicate a decrease in penicillin resistance; except in Germany, where levels of penicillin resistant *S. aureus* has remained at 30-40 % from the 1960s through to the 1990s. In general comparisons between and even within countries are difficult due to the various methods of resistance determination. Sensitivity of *S. aureus* to antimicrobials other than penicillin has remained good over a long period of time (23). Occasionally a dual resistance against lincomycin and erythromycin is detected, but the rate of resistant strains is generally under 10 %. Sometimes in strains of *S. aureus* isolated from bovine mastitis minimal inhibitory concentrations high enough to qualify them as oxacillin-resistant are observed. However, the general opinion is that the mechanism of resistance is probably due to hyperproduction of β-lactamase rather than to the altered penicillin-binding protein found in human strains of MRSA (methicillin-resistant *S. aureus*). *S. aureus* isolates are some-

what site specific, and not all strains are equally capable to cause IMI. MRSA strains of human origin are obviously unable to adapt to the circumstances in the bovine udder and there are no indications that MRSA strains are momentarily involved in bovine mastitis (24).

We can conclude that there seems to be no urgent need for new antibiotics for treatment of *S. aureus* mastitis. With exception of penicillin in β-lactamase-positive strains, all products currently available on the market have sufficient potential.

Therapy of infectious disease should either assist host defenses in eliminating invading pathogens or reduce pathophysiologic consequences of infection without degrading host defenses. Logically, emphasis in mastitis therapeutics has focused on the elimination of pathogens by use of antimicrobial agents. Also in the control of *staphylococcal* mastitis antibiotic therapy still play an important role (25). Despite of a variety of effective antibiotics, success of treatment of *S. aureus* mastitis particularly during lactation is disappointing (26). Among veterinarians and dairy farmers, therapeutic success is often measured by evaluating reduction of clinical symptoms (clinical cure). However, for long-term effects, total elimination of the pathogen from the gland (bacteriological cure) should be achieved.

In the last decades several antibiotic preparations have been introduced for the treatment of this disease, but a "problem solving drug" has not been invented. So in the majority of mastitis cases where treatment with antibiotics is indicated, benzylpenicillin may still be the drug of choice.

Penicillin has several advantages in the treatment of mastitis:

The minimum inhibitory concentration (MIC) of benzylpenicillin for sensitive mastitis pathogens is ten times lower than in other antibiotics.

Resistance seems not to increase in line with the use of penicillin. The percentage of penicillin resistant staphylococci is lower in Scandinavia than in many other countries despite the fact that the majority of antibiotic-treated mastitis cases in Scandinavia are treated with penicillin.

The pharmacokinetic and –dynamic properties of penicillin are suitable for the treatment of mastitis. Benzylpenicillin is chemically a week base and it distributes well to the mammary tissue and becomes trapped in the milk phase. The half live of penicillin is long enough to allow once a day treatment.

Penicillin has low tissue irritation, which is an advantage especially in the intramammary application.

Penicillin is an environmentally safe substance. As a narrow spectrum antibiotic, which is inactivated by enzymes, penicillin is potentially less harmful for the environment than for instance fluoroquinolones and tetracyclines (27).

Early detection and treatment of *S. aureus* IMI has a considerable impact on the success rate. Treating cows within the first 30 days of infection may offer a 70-80 % cure rate. Every month treatment is delayed; the chance of a cure drops by 20 %. Even dry cow therapy is therefore often ineffective at curing existing *S. aureus* infections (28). The method of administration of antibiotics (intramuscular and/or intramammary) is also of influence for the outcome of the therapy. In many studies it has been described that a combination of an intramuscular and intramammary treatment of clinical and subclinical *S. aureus* mastitis was superior to intramammary treatment alone. It was also shown that the success rate increases as the length of treatment increases. Results of research (9) support the concept that extended antimicrobial therapy is significantly more effective at eliminating natural and experimental IMI than standard treatment regimes.

## Prevention and control

Mastitis is an extremely difficult disease to control because several different microorganisms can invade the udder, multiply there and produce harmful substances that result in inflammation. Microbes that most frequently cause mastitis can be divided into two categories: contagious pathogens that are spread from cow to cow, primarily during the milking process; and environmental pathogens that are found throughout the environment of dairy cows. Current mastitis control programs, which were devised in the 1960s, are based on hygiene including teat disinfection; antimicrobial therapy and culling of chronically infected cows. Acceptance and application of these measures throughout the world has led to considerable progress in controlling mastitis caused by *Streptococcus agalactiae* and to a much lesser extent *S. aureus* (29). This failure can be partly explain by ecological observations of *S. aureus* infections, which indicates that the udder of the adult cow is not the only reservoir of the organism and that transmission is not necessarily limited to the milking process. Therefore sources other than infected udder of lactating cows are likely involved in the epidemiology of *S. aureus* IMI in the dairy herd (30). Recent studies (3) have provided some evidence that substantial variation in epidemiology exists within one bacte-

rial species. Also in case of *S. aureus* there is a large variation in the genome of individual strains and it seems, that also very different clinical patterns emerge from these strains. A better understanding of strain-specific epidemiology within bacterial species will consequently have a major impact on the specific control strategies that are successful to prevent or at least reduce IMI in herds. Experiences from countries where *S. aureus* udder infections were significantly reduced in the last decade indicate that culling of chronically infected cows is the most powerful tool to achieve this goal. However, especially in smaller herds, where culling for mastitis is limited to two or three animals per year, elimination of infected cows alone will not solve the problem. In such cases, beside general accepted preventive measures, which are directed to reduce the spread of *S. aureus* during milking, herd-specific factors should be recognized. In herds where culling of all infected cows is not possible segregation of infected cows or using separate milking clusters on infected cows is a viable option. Smaller herds can designate one or two milking clusters as "Staph" units. These claws should be clearly marked and only used on infected cows. Another option is to milk the uninfected cows first and the "Staph" cows last. This method relies on the post-milking sanitation procedures to effectively remove potential udder skin contamination. Larger herds can create a "Staph" milking group. Even though these cows are housed in the same free stall barn, they can be separated at milking time and milked last. Heifers and new herd additions can be potential sources for introduction of *S. aureus* into uninfected herds. Therefore all new herd additions including heifers should be cultured within 30 days of entering the lactating herd (29).

## Conclusions

Understanding the epidemiology of a disease, including disease distribution and transmission, is important for the development of prevention and control programs. Procedures that may be very successful in control or eradication of contagious mastitis, may not be effective in the control of environmental mastitis, and vice versa .

The best way to control *S. aureus* mastitis in a dairy herd is to identify infected cows and prevent the exposure of healthy mammary glands to the pathogen. Elimination of existing infections is best achieved with an appropriate therapy regime during the lactation period; complete dry cow therapy and culling chronically infected cows. New infections can be prevented by proper milking time procedures, post-milking teat dipping, maintaining excellent teat skin condition, and segregating infected cows.

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## *STAPHYLOCOCCUS AUREUS* - ALI RES MORAMO ŽIVETI Z NJIM?

A. Pengov

**Povzetek:** *Staphylococcus aureus* (*S. aureus*) še vedno sodi med najpogostejše povzročitelje vnetja mlečne žleze po svetu. Programi za zmanjševanje števila mastitisov, ki jih povzroča *S. aureus*, temeljijo predvsem na preprečevanju novih okužb in ne na zdravljenju obstoječih vnetij mlečne žleze. Navkljub znatnemu napredku na tem področju, pa predstavljajo mastitisi, ki jih povzroča *S. aureus* za posamezne rejce veliko finančno breme. Odpornost na penicilin se je med sevi *S. aureus* izoliranimi iz mlečne žleze od leta 1965 ko je znašala približno 20 % hitro povečevala in v sedemdesetih letih dosegla 45 %. V zadnjem obdobju pa smo priča ponovnemu padcu odpornosti *S. aureus* na penicilinske preparate, ki je danes približno 30 %. Čeprav je terapevtska vrednost penicilina v mnogih državah omejena, pa je na tržišču dovolj učinkovitih preparatov za zdravljenje stafilokoknih mastitisov. Sevi *S. aureus* odporni na meticilin (MRSA), ki jih v zadnjih letih vse pogosteje povezujemo z okužbami pri ljudeh, vsaj zaenkrat ne povzročajo mastitisa pri govedu. Pri sanaciji problematičnih čred je potrebno upoštevati tako dejavnike povezane z zdravljenjem obstoječih okužb, kot tudi mere za preprečevanje novih primerov stafilokoknega mastitisa.

**Ključne besede:** govedo, bolezni – preprečevanje in nadzor; mastitis, bovini – zdravljenje z zdravili; *Staphylococcus aureus*; krave

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**Review Paper**

# SURGERY OF UMBILICAL CORD REMNANTS IN CALVES

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**Summary:** Umbilical remnants in calves may be diagnosed by thorough clinical examination of the umbilicus and the general status. Ultrasonography, using a 5 MHz probe, represents the most accurate imaging technique for preoperative differentiation of the size and delineation of intraabdominal umbilical remnants. Therapy of such conditions consists of laparotomy and excision of the affected structures in toto under local or general anaesthesia with the animal in dorsal recumbency. Prognosis is generally good except for cases with spread of infection into other organs and infections of the umbilical arteries that extend to far towards the abdominal aorta.

**Key words:** catlle diseases; umbilicus – ultrasonography; umbilical cord – abnormality – surgery; infection – pathology; treatment outcome; cattle

## Introduction

Umbilical cord remnant infections include omphalophlebitis, omphaloarteriitis, and infection of the urachus. *Arcanobacterium pyogenes* is the most commonly isolated microbe from infected umbilical cord remnants. In about a fourth of the cases, infection of umbilical cord remnant occurs concurrently with umbilical hernia. The prevalence of umbilical infection is judged to be 5%. The most important predisposing factors include insufficient hygiene at birth and thereafter and failure of passive transfer.

## Diagnostic procedures

The following techniques are routinely used to differentiate among umbilical pathologies: Thorough general clinical examination with special emphasis on palpation of the joints, visual inspection and palpation of the external umbilicus, bimanual palpation of the intraabdominal umbilical structures in the sedated animal, puncture, and ultrasonography. The latter is the most time consuming diagnostic procedure, but allows for the most accurate diagnosis (1,2). Probing of a fistulating process is possible, but may only be performed with special care in order not to perforate the wall of the umbilical remnant.

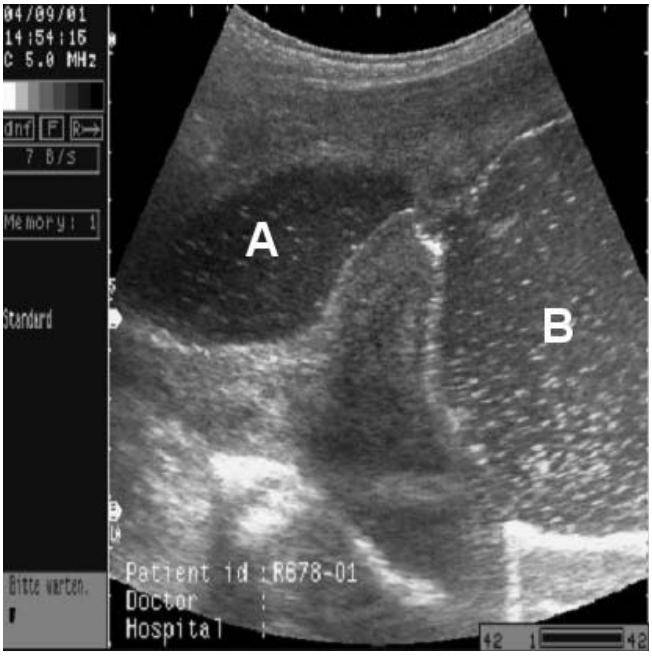


**Figure 1:** Umbilical Sonography
A= urinary bladder
B= urachal abscess

Ultrasonography is indicated for diagnosis of intraabdominal umbilical cord remnant infections; it allows differentiation of processes affecting umbilical arteries, the umbilical vein, and the urachus. Furthermore, the extent of the septic process may well be defined, such as potential involvement of urinary bladder and liver. The

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examination is performed in the standing animal with the examiner positioned to right of the calf. The region delineated cranially by the xyphoid and caudally by the teats/scrotum must be clipped, cleaned with water, and contact gel is administered. Using a 5 MHz probe, the areas cranial and caudal to the umbilicus are meticulously scanned for the presence of intraabdominal umbilical cord remnants (Fig. 1; A = urinary bladder; B = urachal abscess). Alternatively, a 7.5 or 8 MHz transrectal probe may be used. Because of the limited depth of penetration of such probes, imaging of structures in the depth of the abdomen i.e. liver abscesses or the course of infected umbilical arteries in the area of the urinary bladder may no be possible. Physiologic involution of intraabdominal umbilical structures allows identification of the umbilical vein for no longer than 3 weeks and of the umbilical arteries for no longer than 10 days after birth (2).

## Surgical procedures

Depending on the age, animals are kept off milk and roughage for at least 12 hours prior to surgery, and Sodium-Penicillin (30,000 IU /kg of bodyweight) is administered intravenously at 1 to 2 hours before surgery. The intervention is performed either under deep sedation and local, including lumbosacral epidural anaesthesia (3), or under general intravenous or inhalation anaesthesia. Calves are positioned in dorsal recumbency, umbilical fistulas tightly sutured, and the surgical field is prepared for aseptic surgery. An elliptical incision of the umbilicus at its base is performed, and the abdominal cavity is opened starting lateral to the umbilicus. The infectious process is bluntly dissected from adhered tissues such as the greater omentum and/or intestinal structures and excised in *toto*. If the urinary bladder is involved into the process, partial resection of the bladder is indicated. The bladder is sutured with 2 seromuscular inverting continuous sutures, using 3-0 monofilament absorbable suture material. If the liver is involved, marsupialisation of the umbilical vein abscess is indicated (4,5). We do not advise to perform partial liver resection in such cases, because severe bleeding represents a serious complication. The abdominal wall is closed routinely: The peritoneum and the rectus sheaths are approximated with one interrupted cruciate suture pattern, using size 2 PDS suture material. The subcutaneous tissue is sutured with one or two continuous sutures, using absorbable material (metric 7 chromic catgut) and the skin with a Ford-interlocking suture, using nonabsorbable suture material. Laparoscopically assisted resection of umbilical cord remnants has been described in foals (6) and experimentally in healthy calves (7).

## Postoperative care and prognosis

Antimicrobials are administered for at least 3 days postoperatively, and the calf should be confined to a box stall for at least 1 month in order to minimize the chance of wound dehiscence. Analgesics are not routinely administered. Prognosis is good except for abscesses of the umbilical arteries that extend too far towards the abdominal aorta. If spread of the umbilical infection has occurred into other organs such as the liver (multiple abscesses), the heart, or multiple joints, the prognosis is poor, and euthanasia of the calf should be considered.

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## KIRURŠKO ODSTRANJEVANJE OSTANKOV POPKOVINE PRI TELETIH

A. Steiner

**Povzetek:** Ostanki popkovine pri teletih se diagnosticirajo s kliničnim pregledom področja popka in splošnega stanja živali. Najboljše diagnostično sredstvo za predoperacijsko določanje velikosti in opredelitev intraabdominalnih ostankov popkovine je ultrazvočni pregled s 5-megaherčno sondo. Zdravljenje teh stanj je kirurško. V hrbtnem položaju živali z lokalno ali splošno anestezijo opravimo laparatomijo in izrežemo vso prizadeto tkivo. Prognoza je v večini primerov dobra, razen kadar se okužba razširi na druge organe oz. se okužba popkovnih arterij razteza predaleč k abdominalni aorti.

**Ključne besede:** govedo, bolezni; popek – ultrasonografija; popkovina – nepravilnosti – kirurgija; okužba – patologija; zdravljenje, izid; govedo

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**Original Research Paper**

# PULPITIS AND PULP NECROSIS AS A SEQUEL TO PERIODONTAL DISEASE IN DOGS

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**Summary:** There is general agreement that pulpal disease can initiate and/or perpetuate periodontal disease; the opposite theory is controversial. Several investigators suggest that pulpitis could be a sequel to periodontal disease. If accessory canals are the main entrance for bacteria and their metabolites to the dental pulp, it is hypothesized that periodontal disease associated pulpitis is less likely to occur in dogs compared to humans, because the typical canine root canal anatomy has very few lateral ramifications away from the delta at the root apex.

The histopathology of dog teeth extracted because of moderate to advanced periodontal disease was studied to determine the presence and range of pulpitis or necrosis. A total number of 22 affected teeth were examined and changes were compared with 5 control teeth obtained from dogs with no clinically detected periodontal disease. There was obvious pulpitis in 27.3 % of periodontally affected teeth with mild inflammation in additional 18.2 %. Pulp necrosis was observed in 40.9 % of cases. Chronic apical periodontitis was confirmed in 44.4 % of teeth with pulp necrosis.

The finding of obvious chronic and acute pulpitis in a significant proportion of cases, despite the low number of lateral canals, was unexpected. Further study is required to determine pathogenesis of pulpitis. The possibility of pulpitis or pulp necrosis in periodontaly involved teeth should be considered when planning periodontal treatment.

**Keywords**: dentistry – veterinary; periodontal diseases; dental pulp diseases – pathology; dogs

## Introduction

Periodontal disease is the most common chronic disease of dogs (1, 2), which locally affects periodontal tissues and leads to teeth loss (1, 3, 4). Additionally, evidence increases, that periodontal disease can be connected to different systemic conditions (2, 5 - 13).

Whilst there is general agreement that pulpal disease can initiate and/or perpetuate periodontal disease (14 - 18), the opposite theory is controversial (15, 17 - 24). Several investigators (4, 25 - 27) suggest that pulpitis could be a sequel to periodontal disease in humans and monkeys as bacteria and their metabolites may gain entry from an infected periodontal ligament through exposed accessory canals. However, this is less likely to occur in dog teeth as compared to human teeth because the typical canine root canal anatomy has very few lateral ramifications away from the delta at the root apex (15, 28 - 30). Dentinal tubules are also wide enough for bacteria and their products to pass into dental pulp tissue (3, 4, 31, 32), but the cementum and enamel play an important defensive role in such cases provided they remain intact (14, 23, 25, 31). Root planing may damage cementum and enhance exposure of dentinal tubules (15, 23) or damage blood vessels entering the pulp through the lateral canals (18). However, Bergenholtz and Lindhe (25) suggest that root planing has no effect on pathology of dental pulp. Bacteria and their products can invade the dental pulp via the blood stream, if sufficient enters the systemic circulation and evades the lymphoreticular filtering system (4, 33).

The aim of the study was to histologically examine the occurence of pulpitis and pulp

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necrosis in dog teeth extracted because of advanced periodontal disease.

## Materials and methods

*Population of dogs*

Client-owned dogs in good general health except for spontaneous periodontal treatment that were presented to the Clinic for Small Animal Medicine and Surgery, University of Ljubljana, between October 2002 and January 2003 for treatment were included in the study. Dogs had not received any professional dental treatment within the previous three years and had received maximum three dental treatments in their lives.

In addition, control teeth, not affected with clinically detected periodontal disease, were immediately post mortem obtained for comparison from two seven- and eight-years-old cocker spaniels that had been euthanised for clinical reasons unrelated to the oral cavity. No previous periodontal treatments were reported for these two dogs.

Dogs were anaesthesied (premedicated with acepromazine 0.02 mg/kg, methadon 0.2 mg/kg and amoxicillin-clavulanic acid 20 mg/kg; induction of anaesthesia was performed with propofol 3 – 4 mg/kg and maintenance with 1 % to 2.5 % isofluorane) and the oral cavity of each dog was then assesed visually and with a William's periodontal probe and radiographed following the clinic's normal procedure.

*Specimens selection*

Teeth included in the study did not have any carious lesions or complicated crown fractures.

Furcation exposure in multi rooted teeth was graded on a 0 to 3 scale, depending on the penetration of the periodontal probe: 0 – none furcation involvement, 1 – lateral exposure, 2 – incomplete penetration, 3 – complete penetration.

Tooth mobility was graded on a 0 to 4 scale as sugested by Rateitschak et al. (34): 0 – physiological, 1 – detectably increased, 2 – clearly visible, 3 – severe, 4 – extreme.

Radiographic examination was performed if clinical evaluation was not diagnostic. Teeth with maximal furcation involvement or extremely mobile teeth were not radiographed.

Treatment included routine periodontal therapy (supra- and subgingival scaling followed by polishing and gingival lavage) and extraction of compromised teeth (primarily those with excessive mobility, furcation penetration, loss of one third or more of their periodontal attachment, or deep periodontal pocketing). Extraction was performed using standard extraction technique (sectioning of multirooted teeth using a cutting bur in a high speed dental handpiece with copious water spray, raising mucogingival access flaps and alveolar bone removal, as required to facilitate use of elevation/luxation instruments) (35).

*Processing of the specimens*

Each sectioned extracted tooth was immediately placed in >15-times its own volume of buffered (pH 7.2) 10 % formalin solution. Where the crowns were intact following extraction a hole was made at the cusp tip, using the high speed handpiece, to assist penetration of formalin into the pulp prior to immersion in fixative.

The extracted teeth were fixed for between one and two weeks during which time they were maintained at room temperature and the formalin changed twice weekly. After fixation the teeth were thoroughly rinsed with water prior to demineralisation in >15-times their volume of 12 % ethylenediamine-tetraacetic acid (EDTA). The teeth were maintained at room temperature with gentle agitation and the EDTA solution was changed every seventh day until two weeks after there was no radiographic indication of remaining mineral content. Following demineralisation the teeth were rinsed three times in distilled water and re-fixed for at least three days in buffered formalin prior to further processing.

The fixed, demineralised teeth were processed and embedded in wax in the mesiodistal or buccolingual plane for histological sectioning at thicknesses of 5 to 10 microns. Sections were adhered on glass slides and dried in an oven at 42 şC for 24 hours before being routinely stained with haematoxylin and eosin and mounted under a cover slip. Mesial and distal roots (palatal root being appropriate just in one case) of multirooted teeth were processed and examined.

Each sample of control and diseased teeth was serially examined by light microscopy (the same examiner for all specimens NA), presence of inflammatory cells characteristic for acute and chronic inflammation being recorded in odontoblast layer, in pulp blood vessels and outside blood vessels. Changes in blood vessels were also recorded and photomicrographs were obtained of representative sections. The pulps were then scored on a 0 to 3 scale (0 – no changes, 1 – mild changes, 2 – moderate changes, 3 – severe changes), which is based on the protocols for histological classification of pulp diseases used by

Czarnecki and Schilder (22). Pulps were then evaluated regarding histological evaluation of the pulp described by Seltzer and Bender (4): intact uninflamed – unaltered cells, minimal quantity of collagen fibres, normal blood vessels, altered uninflamed – no inflammatory cells or some present in blood vessels with no margination, altered odontoblast layer/predentin, abnormal disposition of dentin, altered blood vessels, increased amount of secondary/reparative dentin, increased collagen fibres amount, transitional stage – polymorphonuclear (PMN) cells in blood vessels with/without margination, some plasmacells in pulp tissue (not infiltrates), blood vessels/capillary congestion, inflamed pulp – acute pulpitis – increased numbers of PMN cells in blood vessels with margination, some plasma cells may be in pulp tissue, inflamed pulp – chronic pulpitis – increased numbers of plasma cells in tissue – if infiltrates then *partial chronic pulpitis*/if scattered then *total chronic pulpitis*, total necrosis – no structures distinct in the pulp chamber.

Changes in the pulp related to pulp exposure during or immediately after extraction were assessed but excluded from the scoring.

*Classifying specimens*

Each tooth was classified according to the extent and nature of its periodontal disease involvement to enable investigation for correlations between periodontal parameters and histological findings.

According to probing depth teeth were classified in four groups as suggested by Rubach and Mitchell (27): A: >0 -2 mm, B: 3 – 5 mm, C: 6 – 8 mm, D: >8 mm.

According to furcation involvement teeth were classified in four groups: 0: none furcation involvement, 1: lateral exposure, 2: incomplete penetration, 3: complete penetration.

According to tooth mobility five groups were formed: 0: physiological mobility, 1: detectably increased mobility, 2: clearly visible mobility, 3: severe mobility, 4: extreme mobility.

*Statistical analysis*

The results were tested for correlations with periodontal parameters using univariant analysis and test for normality, Fisher's exact tests and Spearman's correlation coefficient using a commercial statistical software package (SAS 8.01, procedures MEANS, FREQ and CORR), values of $P<0.05$ being regarded as significant.

## Results

*Population of dogs and teeth included in the study*

Nine small and medium-sized dogs (5 males and 4 females) aged 5 to 12 years (mean 8.8 years) were finally included in the study. Six dogs had had no previous dental treatment, 3 others had had maximally 2 previous dental treatments in their life, last at least 3 years ago. Statistical correlation was positive between age and number of previous dental treatments.

45 teeth were extracted during the study because of advanced periodontal disease (Figure 1) with left mandibular first molar being overrepresented (11.1 %).

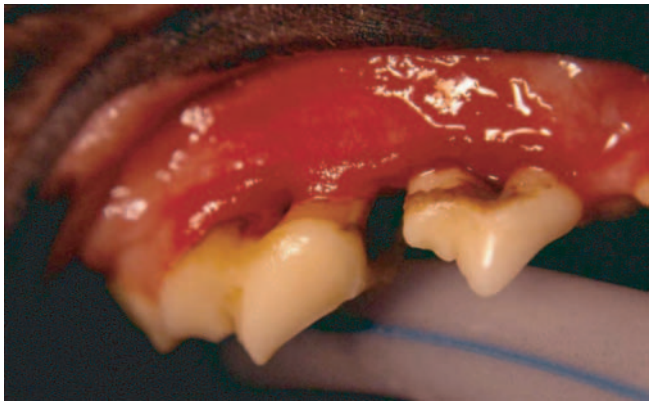

**Figure 1:** Advanced periodontal disease in a 10-year-old male cocker spaniel. Furcation exposure (F3)

Final histological examination was done in 22 for the evaluation appropriate teeth. 23 teeth were excluded from the study as the pulp tissue was too small or severely damaged during processing, which would make evaluation of the pulp unreliable. Main observations regarding the population of dogs, affected teeth and affected periodontal tissues and histological evaluation of the pulp are presented in Table 1.

*Periodontal tissues affection*

Only 18.2 % of the teeth had normal probing depth and all of the teeth showed some degree of mobility with 63.7 % showing severe or extreme mobility. The majority of teeth (88.9 %) had furcation exposure (Figure 1).

**Table 1:** Characteristics of population of dogs, affected teeth, affected periodontal tissues and histological condition of the pulp. Control teeth are not included.

| DOG | AGE | PREVIOUS TREATMENTS | TOOTH | PERIODONTAL INVOLVEMENT | | | HISTOLOGIC EVALUATION OF THE PULP |
|---|---|---|---|---|---|---|---|
| | | | | PD | M | F | |
| 1 | 12 | 2 | right mandibular canine | D | 4 | / | total necrosis |
| | | | left mandibular first molar | B | 4 | 3 | chronic partial pulpitis |
| | | | left maxillary canine | C | 3 | / | total necrosis |
| | | | right mandibular fourth premolar | A | 4 | 2 | total necrosis |
| | | | right mandibular first molar | B | 4 | 3 | chronic total pulpitis |
| 2 | 10 | 1 | left mandibular first molar | B | 3 | 3 | total necrosis |
| | | | right maxillary third incisor | B | 3 | / | altered uninflamed |
| 3 | 8 | 0 | left mandibular canine tooth | B | 3 | / | altered uninflamed |
| | | | left maxillary fourth premolar | B | 3 | 3 | total necrosis |
| | | | right mandibular first molar | B | 3 | 3 | total necrosis |
| | | | left mandibular fourth premolar | A | 3 | 3 | acute pulpitis |
| | | | left mandibular first molar | B | 3 | 3 | acute pulpitis |
| 4 | 9 | 0 | right maxillary first molar | B | 3 | 3 | chronic total pulpitis |
| 5 | 5 | 0 | right maxillary first molar | D | 3 | 2 | total necrosis |
| 6 | 7 | 0 | right maxillary third premolar | B | 2 | 3 | transitional stage |
| 7 | 8 | 0 | left maxillary second premolar | B | 2 | 3 | transitional stage |
| | | | right maxillary second premolar | B | 2 | 3 | altered uninflamed |
| 8 | 10 | 0 | left maxillary third premolar | A | 1 | 3 | total necrosis |
| | | | left mandibular first molar | A | 1 | 3 | total necrosis |
| 9 | 10 | 2 | right maxillary fourth premolar | B | 1 | 3 | transitional stage |
| | | | right mandibular first molar | B | 1 | 3 | transitional stage |
| | | | left mandibular first molar | B | 1 | 3 | chronic partial pulpitis |

Legend: D (probing depth) : A: >0 -2 mm, B: 3 – 5 mm, C: 6 – 8 mm, D: >8 mm,
M (mobility): : physiological mobility, 1: detectably increased mobility, 2: clearly visible mobility, 3: severe mobility, 4: extreme mobility,
F (furcation involvement): 0: none furcation involvement, 1: lateral exposure, 2: incomplete penetration, 3: complete penetration

*Histologic evaluation of the pulp*

Findings in control teeth:

Figure 2 illustrates what was considered "normal" pulp in aged animals. In all pulps observed blood vessels were mildly (graded 1 to 2) dilated, but capillary congestion was seen only in the crown part of one root; in this case increased number of inflammatory cells were also present in blood vessels (Figure 3). No chronic apical periodontitis was seen in any case.

Findings in periodontally affected teeth:

Intact uninflamed pulp was not observed. Altered uninflamed pulp was diagnosed in 13.6 % of the teeth. Pulpitis was diagnosed in 27.3 % of cases with additional 18.2 % of the teeth showing increased numbers of PMN cells in blood vessels with margination in some parts of the blood vessels, where the pulp was diagnosed as being in transitional stage. Necrosis was found in 40.9 % of cases and in 44.4 % of these cases chronic apical periodontitis was confirmed (only in one case both roots were affected).

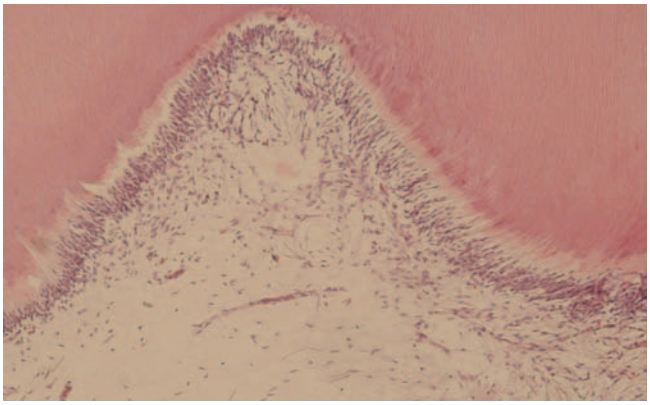

**Figure 2:** "Normal" pulp tissue of aged dogs. (Light microscope. Mag. 100X, haematoxylin and eosin)

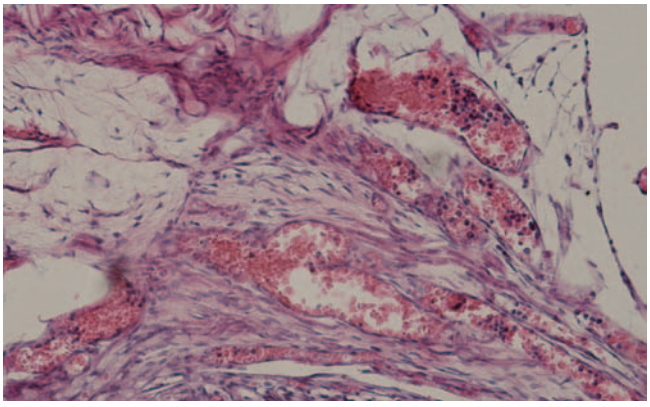

**Figure 3:** Blood vessels congestion and PMN cells present in the blood vessels in one control tooth. (Light microscope. Mag. 200X, haematoxylin and eosin)

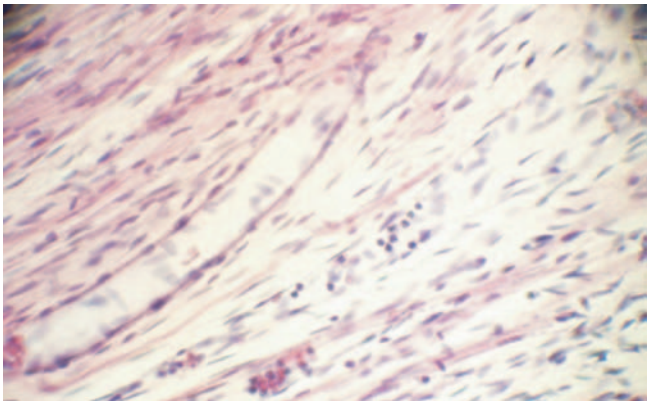

**Figure 4:** Acute pulpitis. Increased number of PMN cells in blood vessels. PMN cells margination. (Light microscope. Mag. 250X, haematoxylin and eosin)

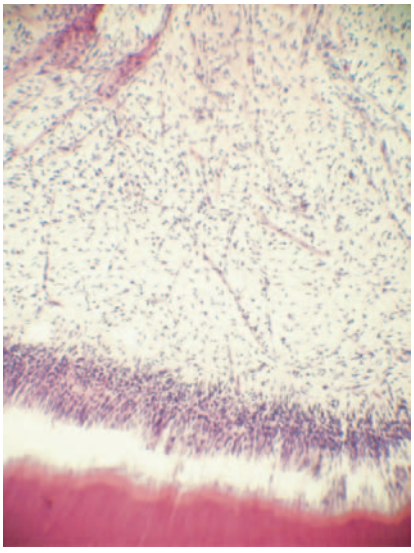

**Figure 5:** Congested capilaries in crown portion of the pulp in acute pulpitis. (Light microscope. Mag. 100X, haematoxylin and eosin)

Acute pulpitis was confirmed in 9.1 % of the teeth, these teeth having increased numbers of PMN cells in the blood vessels. Margination of PMN cells was observed (Figure 4) and blood vessels were dilated and congested (Figure 5). PMN cells were the main cells involved in acute pulpitis but were not observed outside the blood vessels.

Chronic pulpitis was diagnosed in 18.2 % of cases. In half (9.1 %) of these cases plasma cells, the main cells observed in the cases of chronic pulpitis, were located in the crown portion of the teeth examined. However, some PMN cells were also observed in blood vessels. In these cases pulpitis was determined as chronic partial pulpitis. (Figure 6)

In 9.1 % of cases chronic total pulpitis (Figure 7) was diagnosed as plasma cells were scatered through the pulp and PMN cell number in blood vessels was increased.

The degree of pulpitis varied among the tooth roots in some cases of multirooted teeth.

Some degree of dilatation of blood vessels was observed in all the vital teeth (13 teeth) and in some cases hyalin and/or fibrin was seen in the blood vessels (Figure 8).

There was no statistical correlation found between frequency of previous dental treatments and histologic findings within dental pulp. However, a small number of specimens limited the reliability of statistical tests for correlation between periodontal tissues affection and histologic findings within the dental pulp

## Discussion

Inflammation of pulp tissue as well as healing is comparable to that of connective tissue else-

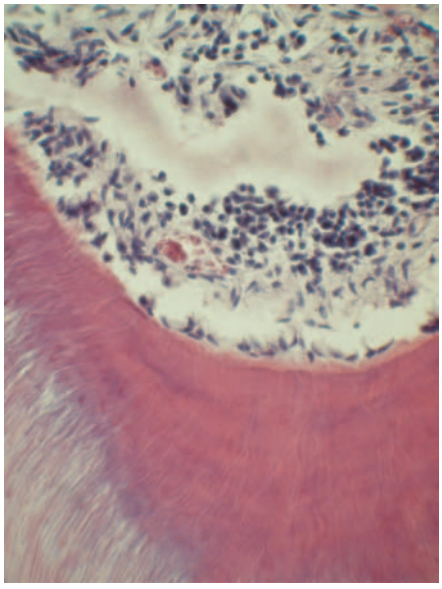

**Figure 6:** Plasma cells in crown portion of the pulp. Chronic partial (crown) pulpitis. Reparative dentine apposition. (Light microscope. Mag. 250X, haematoxylin and eosin)

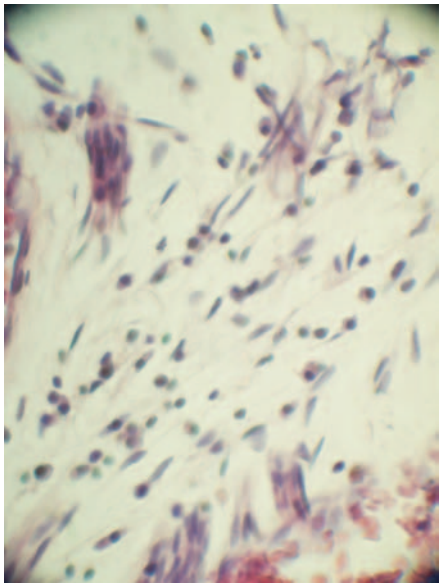

**Figure 7:** Plasma cells scatered through the pulp in chronic total pulpitis. (Light microscope. Mag. 400X, haematoxylin and eosin)

where (36, 37), despite its location in low-compliance pulp chamber (36, 38). The two key components in pulpal inflammation are microcirculation and sensory nerve activity, which effects the pulpal blood flow (38, 39). Bacteria and their products are the main etiological factors for dental pulp inflammation (37), additionally host

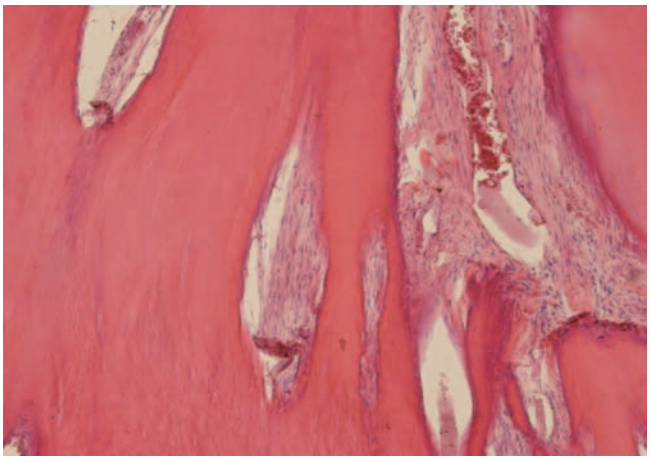

**Figure 8:** Dilatation and congestion of blood vessels, hyalin accumulation in blood vessels in apical delta. (Light microscope. Mag. 100X, haematoxylin and eosin)

response may contribute to the destruction of pulpal and periapical tissues (4, 40, 41).

The greatest difficulty from a clinical point of view is to assess changes and vitality of the pulp (36, 42). If a cause of pulpitis is quickly removed, the pulp tissue can heal, otherwise the pulpitis becomes irreversible with subsequent necrosis and spreading to periapical tissues (4, 31).

Pulpitis as a consequence of periodontal disease was observed in some studies (4, 25 - 27) in human dentistry. However, pulpitis was not confirmed in periodontally affected teeth of dogs (43) and it is also less likely to occur in dogs compared to humans (15, 28 - 30), if accessory and lateral canals are supposed to be the main entrance for bacteria form infected periodontal ligament to the pulp (4, 17). Despite that, pulpitis was confirmed in our study in 27.3 % of cases.

In acute pulpitis PMN cells were the most prominent cells observed, in severe cases margination – sticking of PMN cells to the endothelial lining, was seen, indicative of chemotactic agents present in the pulp tissue (4, 40, 44). PMN cells play an important role in the defence against bacteria in pulpal tissue (45). They contain antibacterial and enzymatic basic proteins that digest the irritant (4, 40) but these substances and the oxidative burst during PMN cells activity can itself cause greater tissue damage than microorganisms (40). However, acute inflammation occurs soon after the injury but persist for a short period - up to a week, then going into a chronic stage, if it is not resolved, and plasma-cells become responsible for humoral immunity (4).

Partial chronic pulpitis in our study was always present in crown portion of the teeth, which could be due to bacteria invading the pulp through a lat-

eral canal in the furcation area of the tooth. Lateral canals in this area are observed in human teeth but not confirmed in dogs (28). Partial chronic pulpitis in these cases, however, is more likely to be a consequence of greater forces acting on these teeth (4) as in both cases it was mandibular left first molar toot that was affected. The finding could also be due to wearing of the crown enamel exposing dentinal tubules (31) as in one case reparative dentin was seen in pulp chamber. Chronic pulpitis, however, is a consequence of a long-acting but moderate irritation (4).

The degree of pulpitis varied between the roots of some multirooted teeth, as has been described by Langeland et al. for humans (26). This is most likely the result of inflammation extending through the pulp from the primary in one root to the other, there being a reduced stimulus away from the primary site. In the same manner chronic apical periodontitis observed only in one root of multirooted teeth can be explained.

Total necrosis of the pulp could be the result of bacteria and/or their metabolites entering the pulp, as well as of reduced nutrition of the pulp due to blood vessel damage (4, 46). Blood vessels in dogs are very fine as they need to pass through apical delta foramina, most of which are under 100 µm in width (28 - 30), comparing to humans, where the apical foramen is reported to measure between 180 and 290 µm (47). The integrity of these blood vessels is easily damaged and blood supply to the dental pulp reduced. Therefore, the high incidence (40.9 %) of pulp necrosis is likely to be related to the high incidence (63.7 %) of increased tooth mobility in advanced periodontitis in dogs. Additionally, necrosis could also be the result of lasting increases in pulp tissue pressure, which is greater in acute inflammation compared to chronic (4). Necrosis as a consequence of advanced periodontal disease is also reported by other investigators (4, 27).

Chronic apical periodontitis occurs as a consequence of bacterial infection of the dental pulp (31, 48) and was only seen in cases of total pulp necrosis. It is the attempt of the body to limit the spreading of destructive processes from the pulp to periapical tissues (48).

Some degree of dilatation of blood vessels was observed in all pulps of control and periodontally affected teeth. This dilatation could be indicative of hyperaemia (49) or a sign of a tooth being orthodontically stressed (50), but it could also be found in intact pulps or it is just fictive due to greater proportion of collagen fibers (4). Congested blood vessels were more obvious in cases with pulpitis.

As worn teeth were not excluded from the study it could be, that in the case of acute pulpitis in control teeth, dentine tubules recently provided diffusion channels for noxious substances which diffused inward toward the pulp (4, 31). However, no trauma to this tooth was reported in the anamnesis and as other teeth from the same animal showed no similar changes it is also less likely that there was an unevidenced inflammation affecting general health of the animal. Additionaly, we found no reports on inflammation of dental pulp tissue in aged teeth.

Hyalin and/or fibrin was seen in the blood vessels, always in connection to the congestion of blood vessels and we presume that it is a consequence of the congestion. No simmilar changes were observed in control teeth.

Histologic observations of lateral canals are not exact (25), however, pulp tissue in all observed lateral canals in our study was not inflamed even if pulpitis was diagnosed. It could therefore be, that lateral canals are not the most important entrance for bacteria and/or their products to the dental pulp. The idea is also suggested by Bergenholtz and Lindhe (25).

No correlation was detected between periodontal tissue affection and histological evaluation of the pulp as »mild« pulp changes were observed in some cases where the periodontal tissue affection was severe; similar observations are reported by several investigators (4, 22, 25) for human teeth. Bergenholtz and Lindhe (25) suggest that pulp tissue alterations are more likely to occur with the prolonged duration of periodontal disease rather than to the severity of the disease. Verstraete (51) suggests that periodontal disease that progresses to involve the exposure of lateral canals, cement and open dentinal tubules on exposed root surfaces, or the apex can result in endodontic lesion. However, most authors (18, 26, 32, 43) agree that the complete disintegration of the pulp tissue occurs only if the periodontal disease is so severe that apical foramina are exposed to bacteria from periodontal pockets extending to the apex of the tooth. Indeed, pulp necrosis in our study was always found in cases, where probing depth was graded severe (classified as C or D).

## Conclusions

The finding of obvious pulpitis in a significant proportion of cases, despite the low number of lateral canals in teeth of dogs comparing to humans, was unexpected. If this pulpitis is related to bacterial contamination from infected periodontal ligament, then lateral canals are not the

most important route af access to dental pulp in dogs affected with periodontal disease. Further study is required to isolate and determine the possibility of pulpitis being the result of aging and attrition of the teeth.

Periodontal disease is known to affect distant tissues, in similar manner it could also affect pulp tissue.

The severity of destruction of periodontal ligament does not seem to be related to pathological alterations within the dental pulp tissue. Therefore, the possibility of pulpitis or pulp necrosis in periodontally involved teeth should be considered when planning periodontal treatment

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# VNETJE IN NEKROZA ZOBNE PULPE KOT POSLEDICA PARODONTALNE BOLEZNI PRI PSIH

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**Povzetek:** Bolezni pulpe vplivajo na zdravje obzobnih tkiv, mnenja o vplivu parodontalne bolezni na zobno pulpo pa so deljena. Nekaj raziskovalcev meni, da je vnetje zobne pulpe lahko posledica parodontalne bolezni. Če ob tem verjamemo, da so lateralni kanali glavno vstopno mesto za bakterije in/ali njihove presnovke iz obolele pozobnice v zobno pulpo, je pri psih vnetje pulpe zaradi parodontalne bolezni veliko manj verjetno kot pri ljudeh. Zobje pri psu imajo namreč veliko manj stranskih ramifikacij pulpinega kanala, razen tistih v apikalni delti.

Pulpitis in nekrozo zobne pulpe pri psih smo ugotavljali s histopatološkim pregledom pulp zob, izdrtih zaradi napredovale parodontalne bolezni. Skupno je bilo pregledanih 22 zob, izdrtih zaradi parodontalne bolezni, za primerjavo pa smo uporabili 5 klinično zdravih zob, odvzetih pri psih s klinično zdravimi obzobnimi tkivi. Vnetje zobne pulpe smo diagnosticirali pri 27.3 % zaradi parodontalne bolezni prizadetih zob, povečano število vnetnih celic pa smo poleg tega ugotovili še v pulpah 18.2 % zob. Nekroza pulpe je bila potrjena v 40.9 % primerov. Pri 44.4 % zob, kjer je bila potrjena nekroza, smo ugotovili kronični apikalni parodontitis.

V nasprotju s pričakovanji smo ugotovili akutni in kronični vnetni odziv v zobni pulpi zob, izdrtih zaradi napredovale parodontalne bolezni. Potrebne bi bile nadaljnje preiskave, da se utemelji patogeneza vnetja zobne pulpe. Kljub temu pa je pri načrtovanju zdravljenja prizadetih obzobnih tkiv smiselno upoštevati tudi možnost, da se pri zobeh, prizadetih zaradi parodontalne bolezni, lahko pojavljata vnetje in nekroza pulpe.

**Ključne besede:** zobozdravstvo – veterinarsko; parodontalne bolezni; zobna pulpa, bolezni – patologija; psi

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**Original Research Paper**

# COMPARISON AND OPTIMIZATION OF TWO PCR TESTS FOR IDENTIFICATION OF *SALMONELLA* IN POULTRY FEEDSTUFFS, LIVER AND FAECES

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**Summary:** Classical cultivation method for Salmonella detection is relatively slow, which can sometimes cause serious clinical and economic consequences. The aim of our study was to compare the efficiency of different methods for rapid Salmonella detection in different samples and to optimize the most appropriate method of detection.
With the comparison and optimization of two previously described methods we established a new effective method for the rapid detection of Salmonella in animal tissues, faeces and feedstuffs after the enrichment step. This method, including the initial incubation on the nutrient media, was used to detect Salmonellae in the feedstuff samples that contained as low as 2 CFU of Salmonella before the incubation. After DNA extraction with a commercially available DNA extraction kit and after amplification by a previously described nested PCR we were able to find 175 CFU of Salmonella in a tissue sample without pre-incubation.

**Key words:** microbiology; veterinary; Salmonella – diagnosis – genetics; comparative study; DNA, bacterial – isolation and purification; polymerase chain reaction; feces – analysis; liver – analysis; animal feed – analysis

## Introduction

*Salmonella* infections are of considerable health and hygienic problem worldwide, as the majority of *Salmonellae* are potentially pathogenic for humans and animals. *Salmonella* contributes to great morbidity and also to mortality, particularly in the undeveloped parts of the world. Salmonellosis is a zoonosis that falls under appropriate regulations (1). The animal-to-human transmission is usually due to the consumption of the food of animal origin. Direct human-to-human, human-to-animal and animal-to-human modes of transmission are also possible (2).

*Salmonella*-caused diseases became widespread with the usage of new feedstuffs (fish, bone and meat flour), intensive farm breeding and frequent consumption of frozen half-prepared food. The investigations have shown that the animals and the food originating from the animals (poultry meat, eggs, milk) represent the most important source of infection for humans, even though human-to-human transmission is possible. The ability of *Salmonella* to multiply in food plays an important role (3). *Salmonellae* can survive in feedstuffs for several years (4); this represents a source of infection for animals (5, 6).

*Salmonella enterica* subsp. *enterica* serovars Enteritidis (antigenic formula is: 1, 9 12: g,m: -) and Typhimurium (antigenic formula is: 1, 4, [5], 12: i: 1,2) (7) are among the most frequent agents causing diarrhoea in domestic and wild animals and enteritis in humans and rodents. *S.* Enteritidis is often isolated from poultry meat and eggs and can be also transmitted vertically (8). *S.* Typhimurium is isolated mostly from pigs. Outbreaks caused by multidrug-resistant *Salmonella* strains have been reported (9, 10, 11). In order to control and treat *Salmonella* infections, effective diagnostic and epizootiological methods are needed (12).

The existing standard culture method for the detection of *Salmonella* (13) requires five working days to generate and confirm positive results. It involves pre-enrichment in the buffer peptone water, selective enrichment, plating on the selec-

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tive agar, and subsequent identification by biochemical and serological tests. In the recent years, more rapid and specific PCR methods, based on the DNA sequence of *Salmonella* genes, have been developed to identify or to characterize pure culture strains (14, 15, 16, 17, 18, 19, 20). The aim of this study was to evaluate two previously published PCR methods for the detection of *Salmonella* in food and field samples and, on this basis, to develop a simple PCR-based protocol suitable for routine analysis of viable *Salmonella* in feedstuffs, animal tissues and faeces.

## Material and methods

### *Samples*

*Salmonella*-free poultry feedstuffs ("NSK" for laying hens and "BRO-finišer" finisher for broilers - TMK Ljubljana, Slovenia), chicken liver and faeces, tested at the Institute of Microbiology and Parasitology, Veterinary Faculty of Ljubljana, were used for the present study.

#### *Bacterial strains and preparation of the inoculum*

Microorganisms used in this study were either isolates from the Internal Collection of Veterinary Faculty (ICVF) or reference strains: *S.* Enteritidis (CAPM 5439), *S.* Typhimurium (ATCC 14028), *Proteus mirabilis* (DSM 788), *Klebsiella pneumoniae* subsp. *pneumoniae* (ATCC 51503), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 10536), *Citrobacter* sp. (ICVF) and *Listeria monocytogenes* (Wurzburg 4A).

The cultures from the archive were inoculated on blood agar (BA) and incubated 20-24 hours at 37°C. Single colonies from the pure culture of *S.* Enteritidis and *S.* Typhimurium were then inoculated in tryptic soy broth (TSB) and incubated 20-24 hours at 37°C. Serial ten-fold dilutions of the culture were made in 0.9% saline; the number of the colony forming units (CFU) was evaluated on the colony counting plates (CCP) after 20-24 hours of incubation at 37°C. The number of the cells in the initial suspension was $7 \times 10^9$ CFU/ml. Further dilutions are shown in.

### *Inoculation and pre-enrichment*

The preparation of the samples was done either with (i) direct extraction without prior incubation or (ii) with previous incubation on the nutrient medium specified below.

(i) Three parallels of 0.5 ml of dilutions 7 to 16 (Table 1) of *S.* Typhimurium culture and 0.5 g of liver were added to 4.5 ml of buffered peptone water (BPW). The mixture was homogenized by vortex for 30 seconds and incubated for 20 minutes at room temperature. 1 ml of the homogenate was used for DNA extraction.

**Table 1:** Dilutions of Salmonella culture

| No | Dilution ratio | CFU/ml | CFU/sample |
|---|---|---|---|
| 1 | undiluted | 7,000,000,000 | 3,500,000,000 |
| 2 | 1:10 ($10^{-1}$) | 700,000,000 | 350,000,000 |
| 3 | 1:10 ($10^{-2}$) | 70,000,000 | 35,000,000 |
| 4 | 1:10 ($10^{-3}$) | 7,000,000 | 3,500,000 |
| 5 | 1:10 ($10^{-4}$) | 700,000 | 350,000 |
| 6 | 1:10 ($10^{-5}$) | 70,000 | 35,000 |
| 7 | 1:10 ($10^{-6}$) | 7,000 | 3,500 |
| 8 | 1:10 ($10^{-7}$) | 700 | 350 |
| 9 | 1:2 ($0.5 \times 10^{-7}$) | 350 | 175 |
| 10 | 1:5 ($10^{-7}$) | 70 | 35 |
| 11 | 1:2 ($0.5 \times 10^{-7}$) | 35 | 17.5 |
| 12 | 1:2 ($0.25 \times 10^{-7}$) | 17.5 | 8.75 |
| 13 | 1:2 ($0.13 \times 10^{-7}$) | 8.75 | 4.38 |
| 14 | 1:2 ($0.63 \times 10^{-8}$) | 4.38 | 2.19 |
| 15 | 1:2 ($0.31 \times 10^{-8}$) | 2.19 | 1.09 |
| 16 | 1:2 ($0.16 \times 10^{-8}$) | 1.09 | 0.54 |

(ii) Feedstuffs and faecal samples were homogenized and divided into 25 g portions. Each portion was mixed with 225 ml BPW and homogenized. Three parallels of 0.5 ml of dilutions 10 to 16 (Table 1) of *S.* Typhimurium culture were added to each sample of faeces and feedstuff. The prepared samples of feedstuffs and faeces and the rest of the previously homogenized liver were incubated in BPW for 18 hours at 37°C, inoculated on tetrathionate broth (TTB) and incubated again for 18 hours at 37°C. 1 ml of TTB was used for DNA extraction.

### *DNA extraction*

DNA extraction from the bacterial cultures. DNA was extracted using the simplified boiling method. A loop full of pure culture was suspended in 50 ml of PCR-grade water (Invitrogen, Carlsbad, CA, USA), heated at 100°C for 15 minutes and centrifuged at 12000 rpm for 2 minutes. The supernatant was used as a source of DNA for PCR.

DNA extraction from liver, feedstuffs and faeces. DNA extraction was done using three different

methods: (Ex-A) extraction with saccharose (Sigma-Aldrich, St. Louis, MO, USA), Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and proteinase K (Sigma-Aldrich, St. Louis, MO, USA) (21), (Ex-B) immunomagnetic separation with anti-*Salmonella* Dynabeads (Dynal, Oslo, Norway) and extraction with a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions (22), and (Ex-C) extraction with a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions.

*DNA amplification*

PCR specificity test. The primers were selected according to the available data in the literature (14, 21). Two different PCR tests were used:

(A-1): The amplification mixture and the protocol described by Rychlik et al. (21) were followed without any modifications.

(A-2): A combined test, using the primers described by Aabo et al. (14) and the amplification protocol described by Trkov et al. (22). A 50 ml amplification mixture was used: 25 µl Taq PCR Master Mix (Qiagen, Hilden, Germany), 22 ml of PCR-grade water (Invitrogen, Carlsbad, CA, USA), 50 pmol of each primer (ST11 and ST15, Invitrogen, Carlsbad, CA, USA) and 2 ml of template DNA.

Detection of *Salmonella* in liver, feedstuffs and faeces with PCR. At first, DNA was amplified as described for the specificity test. Later, the reaction mixtures and the protocols were modified as follows.

(A-1m) The protocol of A-1 was modified using touch down PCR. The first 6 cycles consisted of initial denaturation for 1 minute at 94°C, annealing for 1 minute at 62°C (with the subtraction of 1°C in every cycle) and elongation for 1 minute at 72°C. The next 30 cycles consisted of initial denaturation for 30 seconds at 94°C, annealing for 30 seconds at 56°C and elongation for 30 seconds at 72°C. The final elongation was at 72°C for 7 minutes.

(A-2m) Similarly, the protocol of A-2 was also modified. The first 6 cycles consisted of initial denaturation for 1 minute at 94°C, annealing for 1 minute at 63°C (with the subtraction of 1°C in every cycle) and elongation for 1 minute at 72°C. The next 30 cycles consisted of initial denaturation for 30 seconds at 94°C, annealing for 30 seconds at 57.5°C and elongation for 30 seconds at 72°C. The final elongation was at 72°C for 7 minutes.

(A-2mm) In order to improve the specificity of the amplification we further modified A-2m using hot start PCR with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and increased annealing temperatures (for 1°C) were used. The 50 µl amplification mixture consisted of 1.25 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 5 µl 10x PCR buffer with 1.5 µl $MgCl_2$ (1.5 mmol), 0.2 mM each of dNTP (Invitrogen, Carlsbad, CA, USA), 50 pmol of each primer (Invitrogen, Carlsbad, CA, USA), 33.25 ml PCR-grade water (Invitrogen, Carlsbad, CA, USA) and 5 ml template DNA.

The amplification products were separated on 2% agarose gel and analyzed using a visualization system combining transilluminator and camera (Gel Doc 1000, Bio-Rad, Hercules, CA, USA).

*Inoculation of the samples to the solid media*

One loop (approx. 0.01 ml) of the dilution was inoculated on Rambach (RA) and xylose-lysine-deoxycholate (XLD) agars. Suspicious colonies were subcultured on Drigalski agar (DA) in order to obtain pure culture. The cultures from BPW, TTB, BA and CCP were inoculated to selective media (XLD, RA, DA). The colonies were determined serologically with slide agglutination and commercial biochemical test API (bioMerieux, Marcy-l'Etoile, France).

## Results

*PCR specificity test*

All *Salmonella* strains used in our study showed a specific amplification product with the primers described by Rychlik et al. (21) and Aabo et al. (14). The other 6 different bacterial species used to test the specificity of the primers gave negative results.

*Direct detection of Salmonella in liver*

After DNA extraction by Ex-A and amplification by A-1 negative results were obtained after the first amplification, while nested PCR gave positive results for the samples containing at least 3500 CFU/sample.

DNA extraction with the commercial extraction kit (Ex-C) and amplification by A-1m enabled the detection of 3500 CFU/sample after the first amplification, while nested PCR gave positive results for the samples containing at least 175 CFU/sample. The results are shown in Table 2.

Ex-B and A-2 were not used for direct detection of *Salmonella* in liver.

*Detection of Salmonella in liver, feedstuffs and faeces after enrichment*

Detection results of *Salmonella* in liver and faeces of poultry are presented in Table 2.

Due to non-specific amplification products, obtained with all PCR methods, we additionally optimized the PCR tests for the feedstuff samples. An in-house method, including DNA extraction with the commercial kit (Ex-C) and amplification according to the A-2 using Platinum Taq DNA polymerase, was optimized. The obtained PCR results were then compared with the results of the culture method (Table 3).

According to the results of the colony counting the detection limit of the PCR test was 2.19 CFU/sample (Table 3).

**Table 2:** Detection of *Salmonella* Typhimurium in poultry liver and faeces

| | | | Number of positive samples (3 parallels) | | | | | | | | | | Total number of samples |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Dilutions - CFU/sample* | | | *3500* | *350* | *175* | *35* | *17.5* | *8.75* | *4.38* | *2.19* | *1.09* | *0.54* | |
| Detection in liver without pre-enrichment | Ex-A A-1 | 1st amp. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 |
| | | 2nd amp. | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| | Ex-C A-1 | 1st amp. | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 30 |
| | | 2nd amp. | 3 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| Detection in liver and faeces after pre-enrichment | Ex-A and A-1m | | - | - | - | 2 | 3 | 3 | 3 | 3 | 0 | 0 | 21 |
| | Ex-B and A-2m | | - | - | - | 2 | 3 | 3 | 3 | 3 | 0 | 0 | 21 |
| | Ex-C and A-2m | | - | - | - | 3 | 3 | 3 | 3 | 3 | 0 | 0 | 21 |

Legend: 1st amp. = first amplification; 2nd amp. = second amplification; - = not performed

**Table 3:** Comparison of the culture method and PCR for detection of *Salmonella* Typhimurium in feedstuffs

| Sample | CFU/sample | Cultivation | PCR |
|---|---|---|---|
| 1 | 35 | + | + |
| 2 | | + | + |
| 3 | | + | + |
| 4 | 17.5 | + | + |
| 5 | | + | -* |
| 6 | | + | + |
| 7 | 8.75 | + | + |
| 8 | | + | + |
| 9 | | + | + |
| 10 | 4.38 | + | + |
| 11 | | + | + |
| 12 | | + | + |
| 13 | 2.19 | + | + |
| 14 | | + | + |
| 15 | | + | + |
| 16 | 1.09 | - | - |
| 17 | | - | - |
| 18 | | - | - |
| 19 | 0.50 | - | - |
| 20 | | - | - |
| 21 | | - | - |
| 22 | negative control | - | - |

## Discussion

Classical cultivation method for *Salmonella* detection is relatively slow, which can sometimes cause serious clinical and economic consequences. The aim of our study was to compare the efficiency of different methods for rapid *Salmonella* detection in different samples and to optimize the most appropriate method of detection.

In our study previously described methods of cultivation, DNA extraction and amplification (21, 22,) were used and compared. The methods that best suited our needs were optimized with the aim of choosing the method that would be comparable to the cultivation method in the terms of quality and reliability. Considering the fact that the costs of the novel diagnostic methods still exceed the cost of the classical methods, its main advantage was supposed to be the rapidity.

At first, the specificity of the primers described by Czech (21) and Danish (14) authors was tested with different bacterial species: *Escherischia coli*, *Proteus* sp., *Citrobacter* sp., *Klebsiella* sp., *Listeria* sp. and *Salmonella enterica* subsp. *enterica*. The results were comparable with the findings of Lin and Tsen (23). Because of the increasing clinical importance of the two *Salmonella* serovars, *S.* Enteritidis and *S.* Typhimurium, respectively, they were selected for the testing. A variety of different bacteria were used in order to check the methods' specificity for the genus *Salmonella*, while the two different serovars were used to detect any possible differences in the sensitivity between the serovars. All the three primer sets tested in our study were specific for the genus *Salmonella*. The PCR test performed by serial dilutions of the two most common *Salmonella* serovars, isolated in our laboratory, showed no differences in the sensitivity between the serovars. It was concluded that both serovars could be effectively detected by the same PCR method. So, for further studies only *S.* Typhimu-rium were used.

On the basis of these results the PCR test for the detection of *Salmonella* in different samples (liver, feedstuffs, faeces) was assessed and optimized.

For the direct detection of *Salmonella* in liver, nested PCR was inevitable, although the risk of cross contamination with the amplicons of the previous amplification plays a considerable role. Our results generated with the Ex-A and A-1 (detection of 3.500 CFU/ml) were in general comparable with the results of the Czech study (21), but isolation of DNA with Ex-C proved to be slightly more effective (detection of 175 CFU/ml). Since we still wanted to improve the detection limit, we performed the PCR method after the initial incubation also for liver samples.

After initial incubation of the samples (liver, faeces, feedstuffs), the amplification signal was clearly visible after the first run of amplification, therefore nested PCR was not necessary. These methods proved to be highly sensitive, as they were capable of detecting 2 cells in the sample. They are comparable with the method described by Croci et al. (24) who detected 1 to 10 CFU/25 grams. They also proved to be better than the method described by Cheung et al. (25), which detected $1.5\times10^3$ CFU.

The modified DNA extraction method (Ex-B) is time consuming and expensive but equally effective as the other two methods. Ex-A is the cheapest, but demanding and less suitable for standardization. Ex-C proved to be the most suitable for all kinds of samples. It is commercially available, reasonably priced, and easy to perform and standardize. Regardless of the extraction method it was necessary to incubate the samples for 18-24 hours at 37°C in order to get sufficient DNA yield. Amplification of the feedstuffs samples often resulted in non-specific amplification products, regardless of the extraction and amplification method. The problem was solved by using hot start PCR and by increasing annealing temperatures for 1°C.

Probably the most important achievement of the study is the optimized amplification protocol, combined with the usage of Platinum Taq DNA polymerase (A-2mm). This is a highly specific and effective method for the amplification of the DNA, extracted with the commercial kit from any sample, after the initial incubation on the nutrient media. This method was used to detect *Salmonella* in the feedstuff samples that contained as low as 2 CFU of *Salmonella* before the incubation. The results completely matched the results of the culture method and were even slightly better than the results of the similar studies (21, 22). Thus our goal was achieved: an effective and cost-friendly method for the rapid detection of *Salmonella* in different samples was optimized.

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## PRIMERJAVA IN OPTIMIZACIJA DVEH TESTOV S POLIMERAZNO VERIŽNO REAKCIJO ZA UGOTAVLJANJE SALMONEL V ŽIVALSKIH TKIVIH IN VZORCIH IZ OKOLJA

M. Ocepek, M. Pate, J. Mićunović, V. Bole-Hribovšek

**Povzetek:** Klasična gojiščna preiskava za ugotavljanje salmonel je relativno dolgotrajna, kar ima včasih lahko resne klinične in ekonomske posledice. Cilj naše raziskave je bila primerjava uporabnosti različnih metod za hitro dokazovanje salmonel v različnih vzorcih in optimizacija najprimernejše metode. S primerjavo in optimizacijo dveh že poprej opisanih metod smo razvili novo učinkovito metodo za hitro ugotavljanje salmonel v živalskih tkivih, iztrebkih in živilih po prejšnji inkubaciji na obogatitvenem gojišču. S to metodo smo ugotovili salmonele v vzorcih krmil, ki so pred inkubacijo vsebovali samo 2 CFU salmonel. S kombinacijo izolacije DNK s komercialnim kitom in poprej opisane nested PCR pa nam je uspelo ugotoviti 175 CFU salmonel v vzorcu tkiva brez poprejšnje inkubacije.

**Ključne besede:** mikrobiologija – veterinarska; Salmonella – diagnostika-genetika; primerjalna študija; DNA, bakterijska – izolacija in čiščenje; polimerazna verižna reakcija; feces – analize; jetra – analize; krmila – analize

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