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PREDOMINANT BACTERIAL BIOFILM AND EFFICIENCY OF CLEANING AND DISINFECTION IN A DAIRY INDUSTRY

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Summary

Cleaning and disinfection are important tools in maintaining and improving quality of dairy products. In order to assess this protocol, we studied a dairy located in western Algeria. We conducted periodic visits to follow program practices and to complete a comprehensive questionnaire. We collected several swabs samples at different points before and after cleaning in place procedures. The samples were then submitted to microbiological tests in order to evaluate protocol efficiency and to determine the dominant bacteria in different sites before and after pasteurization. Results showed that the cleaning in place practice was followed as recommended by the manufacturer. However, its effectiveness on depletion of bacteria was low, as the reduction of bacteria was comprised between Log 0.41 and Log 0.86. Even after pasteurization, many bacteria were found, mainly *Micrococcus* sp and *Escherichia coli*. Indeed, there is a need to implant a rigorous and efficient HACCP program to ensure a quality product.

Keywords: cleaning in place, evaluation, hygiene, milk, pasteurization

In food-processing industries, adherence of contaminant microorganisms to surfaces led to adverse effects not only on hygiene quality and public health (32) but also to economic losses (13).

Milk, highly perishable item, is subject to contamination by various microorganisms attached to contact surfaces in dairy plants and subsequent biofilm formation poses a secondary contamination risk in milk and dairy products (12, 31). The major sources of this contamination are usually equipment improperly cleaned and sanitized (16).

This explains that in dairy industry the classical operations of cleaning and disinfection are essential parts of milk production. The efficiency with which these operations were performed, affects greatly the final products quality (31, 8).

In Algeria, the implementation of cleaning and disinfection program in dairy industry is a relatively recent practice (4) implemented with the appearance of the first dairy industries during the 70s, knowing that incorrect application of such a program plays an essential role in determining hygienic quality of milk and dairy products.

Moreover, the main part of the demand for milk is covered by imports and the locally produced milk (3 billion liters) (23) is often highly contaminated (11).

Contamination can occur throughout the milk production chain: hygiene in milking, maintaining the cold chain and delivery deadlines (7), cleaning and disinfection of equipment in contact with milk and refrigeration (28).

Therefore, material not properly cleaned and disinfected becomes favorable to microbial biofilm formation which can contaminate milk (22).

This work was undertaken to identify main biofilm bacteria and to evaluate efficiency of cleansing and disinfection practices in a dairy western Algeria.

Materials and methods

Sampling site:

Located at Sougueur (Governorate of Tiaret), the dairy produces recombined milk from powders imported from various countries (Germany, France; Belgium, Argentina), as well as milk from thirty two (32) surrounding cattle farms and transported to dairy by private collectors in isothermal tanks for pasteurization. The total daily production was around 30000 liters including the 5000 liters collected. This study was conducted in June 2016.

Audit of cleaning in place procedure:

Eight periodic visits were conducted to record handling practices of dairy products in dairy as well as to implement and perform cleaning program. A questionnaire was prepared including several parameters following HACCP Guidelines (14); equipment conformity, cleaning and disinfection.

Samples collection:

Several handling sites of dairy products have been sampled using swabbing method before and after cleaning in place (CIP). Sampling sites were tank of raw milk, storage tanks of recombined milk before and after pasteurization, agitator's blades, elbows and packaging machine. Subsequently, the collected samples were kept cool until further bacteriological analysis.

Evaluating the cleaning in place effectiveness (Enumeration of total flora):

Samples were taken and prepared according to ISO 6887-1 (15) using wet swabbing technique on a surface of 10 cm^2 bounded by a sterile template using aseptic technique (9).

These samples were enumerated for total mesophilic flora using Plate Count Agar (PCA) media for 72 hours of incubation at 30 °C according to AFNOR standard (3).

Results were expressed in colony-forming unit by cm^2 (CFU/cm^2). The Log Kill before and after Cleaning in Place (CIP) was determined by using the following equation:

Log Kill = Log N- Log n (1)

Where N is the count of cells of untreated surface (before CIP) and n is the count of cells after sanitizer treatment (after CIP) (34).

Isolation and identification of contaminant microorganisms:

Others swabbing were realized from following sites: raw milk tank, recombined milk storage tanks before and after pasteurization and packaging machine in order to isolate main contaminant bacteria.

Colonies were identified and biochemically characterized using standard and conventional microbiological techniques including Gram stain, catalase and coagulase tests (3). The identification was completed with API 20E and API Staph gallery (BioMérieux, Marcy l'Etoile, France) (25).

Results and discussions

The cleaning in place practice was realized by the same operator, according to following protocol: a pre-rinsing with hot water followed by alkaline cleaning, inter-rinsing then an acid cleaning followed by a finally rinsing (Table 1).

Table 1

Composition and conditions of use of registered detergents

Detergent	Composition	Application	Conditions of application			
			Time (Min)	pH	C (%)	T (C°)
Alcalin Detergent (Proflow®)	Soda rich in chelating and surfactants	Soaking or circulation	15-20	≥12	1.5	60
Acide Detergent (Steriflow-P310®)	Strong acides and surfactants	Soaking or circulation	15-20	≤ 3	1.5	50

C = concentration ; T= temperature

The highest contamination was observed at milk tank (3.92 and 3.85 Log UFC/ml) probably partially explained by the fact this dairy received milk throughout the day that prevented the most effective form of the cleaning (Table 2).

The highest rate of reduction (Log Kill) was recorded in milk tank, due the easy access but also by the fact that cleaning products stay longer in touch with the funds of storage tanks.

Bacteria isolates were mainly present in storage tanks (83.33 %). However, no difference was observed in number of bacteria before and after pasteurization. This clearly reveals that pasteurization was inefficient, probably due the misuse of heat but also cause the inefficacity of the CIP. This indicates that a detailed audit is necessary to undertake to determine exactly the cause of such a risk and to correct critical points.

Table 2

Enumeration of total flora before and after CIP

Sampling site	Total flora before CIP		Total flora after CIP			
	Samples Nb	Log N (UFC/cm^2)	Samples Nb	Log n UFC/ cm^2	Log Kill (Log N-Log n)	Reduction (Log n/Log N)
Raw milk tank	06	3.85± 0.59	03	2.06± 0.39	1.79	0.53
Storage tanks	13	3.92±0.54	09	2.31±0.52	1.61	0.41
Agitator's blades	04	3.30± 0.13	04	2.73±0.62	0.57	0.82
Bends	05	3.69± 0.54	04	2.74±0.23	0.95	0.74
Wrapping machine	02	3.25± 0.07	02	2.81± 0.58	0.44	0.86
Total	30		22			

The study showed composition of the microflora varied according to sampling sites. Isolation and identification of the contaminant germs revealed presence of common bacteria as well as pathogenic bacteria (Table 3).

Evaluation of CIP efficiency:

The results obtained after CIP (2.06-2.81 Log UFC/ cm2) were low compared with those revealed by Ameur et al. (4) (3.7-5.5), however they remain close to those obtained in India where values after CIP ranged from 1.25 Log UFC/cm^2 to 2.23 Log UFC/ cm^2 (4) (Table 2).

The reduction (Log Kill) varied between 0.44 and 1.79, remains close to those obtained by Malek et al. (01.23-2.36) (24) but were very low compared with those obtained by Sharma and Anaud (31) (3.15-5.55) after recommended sanitation.

However, the lowest rates of reduction were observed at packaging machine followed by agitator's blades due to difficulty to clean these parts (10, 20).

The presence of older biofilms led to increased contamination rates of various segments because older biofilms appear to be more resistant to the penetration of the cleaning and disinfection products then younger (6, 21).

It is recommended that an effective sanitizer should reduce the initial planktonic cell count by five or more Log units and attached cell counts by three or more Log units (34, 26). Therefore, these results show undertaken procedures of CIP were less effective, this could be explained by bacteria capacity to form deposits of organic and mineral stain as well as significant presence of biofilm resistant to disinfecting agents (19). Poor water bacteriological quality has effects on the results of the wash of tanks and other utensils (26).

Table 3

Sampling location

Type of microflora	Raw milk tank	Storage tank		Wrapping machine	Total (%)
		Before pasteurisation	After pasteurisation		
	Number of isolates				
Micrococcus sp	02	06	07	01	16 (29.62)
Escherichia coli 1	*	02	06	*	08 (14.81)
Escherichia coli 2	02	02	01	*	05 (9.25)
Staphylococcus xylosus	*	03	02	*	05 (9.25)
Staphylococcus auricularis	*	*	01	*	01 (1.85)
Staphylococcus aureus	*	*	*	*	*
Kluyvera spp	*	01	01	*	02 (3.7)
Shigella sp	*	01	*	*	01 1.85)
Aeromonas gr1	01	*	01	*	02 (3.7)
Aeromonas gr2	*	05	02	*	07 (12.96)
Vibrio fluviatis	01	01	02	*	04 (7.4)
Providencia	*	*	01	02	03 (5.55)
TOTAL	06 (11.11)	21 (38.88)	24 (44.44)	03 (5.55)	54 (100)

*: absence

Isolation and identification of contaminants germs:

Among isolated bacteria *Micrococcus* sp was predominant (29.62%) followed by *Escherichia coli* (24.07%) species while *Staphylococcus auricularis* and

Shigella represent 1.85 %. Other isolates are non-pathogenic. . In this case, certain bacteria can persist, multiply in milk causing both economic and hygienic effects.

Several studies have shown that in dairy environments, the most commonly encountered bacteria belonged to *Enterobacter, Lactobacillus, Listeria, Micrococcus, Staphylococcus, Streptococcus* and *Pseudomonas* genus (31, 35,29,1).

Persistence of *Micrococcus* sp in several dairy equipment can be explained not only by insufficient pasteurization but also by the fact that skin of mammals and food (especially dairy waste) are considered, as the main housing environment of Micrococcus (17, 2).

Several factors are involved in the growth and survival of *Escherichia coli* in dairy products: product characteristics (composition, Aw, acidity), heat treatment applied and initial rate of contamination in the unpasteurized milk (18).

Growth of bacterial biofilm on joints surface can also constitute a contamination source of the pasteurization lines (5). Thus, it can be either a post-pasteurization contamination (manufacturing equipment, personal), or an excessive contamination of the unpasteurized milk.

Several works reveal foodborne pathogens can enter the milk processing equipment by direct contact with contaminants in the dairy farm environment e.g. fecal contamination and udders of infected animal and through the water used in the milking machines what can explain presence of *Shigella* in dairy equipment (27).

Absence of *Staphylococcus aureus* of the surfaces of the dairy equipment is a good sign but does not imply necessarily their absence in dairy products. Indeed, enterotoxins that are remarkably stable can be present, resisting to irradiation, proteolytic enzymes and especially to the heat while the bacteria is destroyed (33). More important, these toxins could form complexes between them or with the food, preventing their detection after heat treatment while their biological activity persists (30).

Conclusions

Cleaning in place procedures in dairy processing aim the reduction or the eradication of microbes in most of the segments of processing lines. This work revealed presence of contaminant germs in dairy equipment even after performance procedures of Cleaning In Place. This indicates either microorganism's persistence in production lines and their capacity to form biofilms resistant to disinfecting agents or protocol of cleaning in place was not respected. Some recommendations were suggested to improve the efficiency of cleaning in place procedure; such compliance with main CIP parameters (temperature, contact time, mechanic action and concentration) as well as the hygiene of the operators,

which must be irreproachable. Further studies are needed in order to estimate biofilms importance in production lines and their resistance to disinfecting agents.

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COMMON PIG DISEASES ON COMMERCIAL FARMS: A REVIEW

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Summary

Pig production on commercial farms is largely burdened by diseases in all production categories, especially in piglets. Health status of piglets is very delicate subject in large herds, in a confined space, considering diseases spreading. Variation of pathogens in pigs is of great importance not only in showing resistance to drugs, but the occurrence of genetic recombination, which affect the clinical signs and course of the disease. These complications make difficult to diagnose the disease and to apply appropriate therapy and prophylaxis. In pig commercial farms in Serbia most frequently encountered are the following diseases: neonatal colibacillosis, endemic disease, necrotic enteritis, circovirus infections, spirochetal colitis, enterohaemorrhagic syndrome, dysentery and respiratory disease complex. In recent years there was a high occurrence of respiratory disease complex (PRDC) which is increasing health problem in all production units. In this review paper, we present our experiences in diseases of different etiology determined on commercial pig farms.

Keywords: E. coli, spirochetosis, pneumonia, diarrhea

In pigs, special importance is attached to infections caused by microorganisms and parasites.

The aim of this paper was to provide an overview of the infections that are present in commercial farms.

Breeding diseases of bacterial etiology

Neonatal colibacilosis

The disease of of digestion organs caused by gastrotoxigenic *E.coli* were diagnostic on commercial farms and in extensive breeding (9, 10). Neonatal colibacillosis is usually caused by the following strains of *E.coli*:0149, 0149, 08, 0147, 0157. This strains can have their antigenic structure F4 (K88) i F5 (K99 (27). It is widely known that the occurrence of neonatal colibacilosis is a result of

interaction between pathogens and inadequate conditions in the farrowing box. In extensive and intensive type of pig farming gastrointestinal disorders associated with the pathogenic bacteria *E. coli* are notable (2). The term "neonatal colibacillosis" implies particular disease in the first week of life, which is notorious by the phenomenon of loose bowels with sudden deaths caused by Enotrotoxigenic *E. coli* (3). Neonatal colibacillosis is the most often caused by the *E. coli* strains: 0149, 08, 0147 and 0157. These strains can address F4 (K88) and F5 (K99) antigens in their cellular wall structure (27). It is widely known that the occurrence of sinusoidal colibacillosis in piglets is a result of interaction of pathogens and inadequate sanitary (43), and temperature conditions (12, 27) in delivery and maternity section of the farm Neonatal colibacillosis appear abruptly within a few hours after birth (8, 27). It is noted that the piglets poorly intake the milk with occurrence of abdominal enlargement as a result of the accumulation of gas in the intestines with occurrence of colic pain, and then there is a profuse watery, yellowish-greenish to reddish diarrhea (6, 27). As the result of rapid dehydration, there is loss of weight with notable convex back line. Our experience shows that the piglets from primipar sows are more likely to catch this disease than the piglets that originate from the polypar sows. As the most common form of neonatal colibacillosis, enterotoxigenic colibacillosis, which can occur in the piglets 12 hours old up to 3 days of life. Such pigs lose their appetite (poorly sucking) or even cease to suck with progression of the disease. Diarrheal discharge is very pronounced on the legs of piglets which are affected by colibacillosis. The color of the excrement may be greyish to whitish - pink (27). There is a possibility of colibascidosis of reared piglets in industrial type farms. Colibacillosis occurs in the first few days of weaning as a result of massive *E. coli* growth. The predisposing factors this disease type are various, most often it's a stress as a result of weaning and mixing piglets from different groups (7, 13, 27).

(O)Edema disease

Edema disease is acute intoxication of the pigs with toxins (verotoxin-angiotoxin). Exotoxin is produced by some strains of E. coli. In the first week of post weaning period, piglets may develop edema disease. It affects the best piglets (14).

Necrotic enteritis

In the first few days' post-partum, rarely in the older piglets there may appear special disease known as hemorrhagic-necrotic enteritis of suckling piglets. The cause of the disease is *Clostridium perfringens type* C, which produce alpha and beta toxin (among others). The disease course is toxiinfection with a rapid onset. Any therapy is just too delayed or is unsuccessful, and attention should be paid to imunoprophilaxsis of pregnant sows with autochthonous or commercial vaccines (21, 43).

Porcine intestinal spirochetosis (PIS)

Porcine intestinal spirochetosis (PIS) or spirochetal colitis is diarrheal disease of pigs that escale with farmers who are trying to stop using prophylactics antibiotic medications. The cause of the disease is *Brachyspira pilosicoli*, and there are also frequent co-combinations with*Yersinia pseudotuberculosis* and *Lawsonia intracellularis*. Clinical manifestations of this disease and pathomorphological findings convince us that exists in our breeds, especially in the post weaning piglets, but we hardly reach evidence (difficult typing and troublesome DNA extraction from fecal matter). The first clinical sign is diarrhea of the appearance of moist cement with the paches of transparent mucus (17).

Dysentery

It is an indisputable fact that this affection of the intestinal tract (primarily *colon*) is one of the most economically important diseases in large agglomerations. The disease is characterized by a strong mucoid-hemorrhagic discharge due to hyper production of mucus, inflammation and necrosis of the mucous membrane of the colon wall. The diarrhea is followed by reduction of weight, dehydration and fatigue or death if there are no therapy procedure started atright time. Primary role in the etiology of the disease is attributed to *Brachyspira hyodysenteriae*, and other bacterial flora, in particular anaerobic bacteria of the genus *Bacteroides, Fusobacterium* and *Clostridium*. It is believed that some aero bacterial species may also contribute to the development of more intensive pathological alterations. First of all, it is thought on bacteria of the *Bacillus* genus. Most of the swine dysentery patients are the most often infected with the age of 7-16 weeks (28).

Actinobacillus pleuropneumonia

Pleuropneumonia caused by *Actinobacillus pleuropneumoniae* may have peracute, acute or chronic properties. Causative agent has 15 serovars described up to now. Our research show that serovar 2 is dominant in our farms. Different serotypesof this bacteria have unique capsular structure, but cross-reactivity stands because some serotypes share the common lipopolysaccharide (LPS)molecules and all have common proteins of the outer membrane (24). The outer membrane of *A. pleuropneumonia* has five major proteins and approximately 15 smaller proteins. Long-term administration of antibiotics to pigs creates conditions multiple resistance to penicillin, ampicillin and oxytetracycline, which is plasmid, coded. Virulence factors of this causative agent includes: capsule, cytotoxins, hemolysins, proteins from the outside membrane and lipopolysaccharides. There is no evidence that any of these virulence factors are coded via plasmid. Previous studies have shown that the capsule plays an important role in the immune system defenses of the host. *A. pleuropneumoniae* are good mitogens, they can activate the complement system by triggering alternative pathway, induct blood clotting which lead to necrosis –the Schwartzman reaction. When purified LPS is inserted into the lungs of the pigs, there is a notable

inflammatory cell infiltration and inhibition of cytokines IL-1, IL-6 and tumor necrosis factor (TNF). In high concentrations of *A. pleuropneumoniae* toxins are cytolyticfor erythrocytes, lymphocytes and macrophages. They form pores in the cell membranes. The sublitic concentrations toxins are affected by the oxidative metabolism of phagocytic cells and they probably have other biological factors. In the active infections of the microorganism, the lungs are rapidly proliferating. Lipopolysaccharides, and possibly cytokines or other factors, stimulate the mobilization of neutrophils causing inflammatory reactions. When found at the site of the infection, neutrophils got destruct and this is responsible for massive and rapid tissue damage. During acute infections, the presence of an intact capsule may provide an immunosuppressive effect. In an animal surviving an acute infection of microorganism, it can be hidden in fibrin, and it can colonize tonsillar crypts. *A. pleuropneumonia* possibly synthesizes fimbriae and / or helper molecules of adhesion. The acute form of the disease is characterized by a severe bleeding and deposition of fibrin in the lungs. Affected animals show symptoms of severe respiratory distress, cyanosis, fever and vomiting (24).

Breeding diseases of viral ethology

Transmissible gastroenteritis (TGE)

Transmissible gastroenteritis (TGE) is a highly contagious disease of viral etiology characterized by primarily small-bowel infection, short incubation, high morbidity in all age categories, and almost 100% mortality in suckling piglets until in the first week of age. Accordingly, TGE is one of the most serious illnesses due to the high mortality rate, lack of therapy and the limited value of the vaccine. Differences in mortality in the age categories are explained by the time needed for the re-epithelialization of the intestinal epithelium. For this in suckling piglets, it takes 8-12 days and 3 to 4 days for growers. Seasonal occurrence of TGE (winter) is conditioned by the viral sensitivity to the sunlight and the close contact of the susceptible animals. After oral infection, TGEV infects the cylindrical cells of intestinal villi in the small intestine. The cells of the Liberkinian crypts do not allow the reproduction of the virus, and their basic function of epithelial renewal is not distorted. The consequence is shortened epithelium villas, thus reducing the ability of the cells to use lactose causing a malnutrinary syndrome. In such a damaged epithelium, the production of enzymes important for digestion is halted, and the devastated epithelium cannot resuscitate food particles. Because of that, the osmotic pressure (primary lactose) in the lumen of the intestine retains the food, and the microbial fermentation even more increases osmotic pressure of the active particles. Further process progradiates with increased amount of carbohydrates, which come to the ileum and colon get fermented by local bacterial flora into the gas and organic acids. Part of this organic acids react with bicarbonates from the colon content, causing the formation of metabolic acidosis. It is also important to say the activity of adenylate cyclase is not enlarged unlike the pathogenesis of

bacterial etiology infection. The stomach acid, as the first barrier, TGEV, overcomes because it's stable at pH 3. The infection of epithelial cells is achieved by its other characteristic, it is resistant to trypsin. It's believed that are front parts of the intestinal tract are without pathological alteration due protective effect of the bile. The incubation is very short, 18 hours to 3 days. This disease is characterized by yellow-greenish diarrhea, rapid dehydration and vomiting, hypo- or agalactia by sows and high mortality rates in piglets. The clinical picture in older categories consists of a diarrhea, which is short. Weight loss ranges from 4up to 10kg per animal. Of the factors affecting the "severity" of clinical symptoms are low-temperatures in the pen, dietary deficiency (especially zinc) or corticosteroid therapy. In the macroscopic pathology finding there are dehydration of the skin, transparency of small intestine, congestion of mesenteric blood vessels, focal hemorrhages in the tissue, as well as urate agglomerates in the medulla.

Some authors also mention the infarct on spleen edges, bleeding on epiglottis, and in elderly animals bleeding on the urinary bladder. Ulcerations of the stomach with fungal isolates are often detected, so it is believed that this infection by fungi is consequence of the priming viral lesion on the epithelium by TGEV. The earliest microscopic changes seem like the loss of intercellular boundaries, eosinophilia of the cytoplasm and degeneration of epithelial cells. In the parenchyma of the kidney, in addition to urate crystals, macrovacuolar degeneration of tubular epithelium of proximal canals is observed. In most cases, this necrosis can be associated with vomiting and diarrhea and are etiologically related to hypokalemia.

The diagnosis of the disease is based on one of the three tests: proving viral antigen, isolation of viruses or the most usual: by finding of specific antibodies in the serum. As in all diseases of viral etiology, causal therapy is not present. Due to the alterative processes on the intestinal epithelium, osmotic pressure of ingested foods parts can stimulate diarrhea. Provide sufficient amounts of water at their disposal with electrolytes or isotonic fluids. Warm objects due to worn down animals or underdeveloped piglets' thermoregulation, and it is believed that hot floors inactivate the virus. Unorthodox approach in cases where TGE occurs, is the artificial infection or "planned infection system" - programmed oral exposure of sows with parts of the infectious material (parts of the piglets' intestines) 15 days before partus. After that, sows acquire immunity, and the antibodies are secreted over the colostrum. IgA antibodies are important here when lymphocytosis of the gut migrates to the mammary gland from where they are secreted. This phenomenon is known under the name of the intestinal-immunoglobulin, or intestinal mammary base. Secretory IgA in the milk provides protection of the digestive tract during the course of continuous sucking. Research has shown that secretory IgA milk need more time to pass along the intestine than interval between the two successive sucking. This ensures the permanent protection of the suckling pigs' intestine, which indicates on the necessity of regular suckling of the piglets to

make the mucosa of the pig bowel constantly covered with sufficient amount of IgA antibodies that will protect enterocytes from infections (lactogenic immunity).

It is considered that presence of SC (secretory component) of IgA protect from enzymatic degradation. IgA molecules are synthesis by plasma cells (lymphocyte transformation) of lamina propria mucosae, and then transported the mucous membrane through epithelial cells (enterocytes) where they are merged with the secretory component. It is necessary to respect the principle of all in all out, and cleansing, washing and disinfection procedures (12, 20, 43).

Porcine epidemic diarrhea (PED)

Porcine epidemic diarrhea (PED) was first described in England in 1971, in fattening pigs. Nonspecific clinical manifestations of PED (watery diarrhea, rapid dehydration and weight loss) are previously described with TGE, but it was clear that causal factor is different (19). During epizootics of diarrhea in Belgium and England 1978, Coronavirus was isolated (35, 45) and the antigen was different from other pig virus of the same family, TGE and pig encephalomyelitis virus (36). It was noticed that this virus is aggravating all the age categories, specially suckling piglets with mortality rate of 100 percent. The presence of PED virus was then recorded in Hungary (23) and Germany (37). Virus soon spread to the Asian continent where it causes great economic damage even today. In Japan's 1996 epidemic recorded a loss of 39000 suckling piglets, while in the recent times according to the number of deaths the Philippines dominate by 60,000 mortality count (29). PED first appeared in The United States in 2013, and so far it registered in 23 federal state swith 2,692 confirmed cases (Wang et al., 2014). In Serbia it's described by Prodanov-Radulović et.al. (39). The PED virus belongs to the coronaviridae family of females and the genus Alphacoronavirus. The virus has a single-chain, positive-oriented RNA molecule. The average virus size is 130 nm. It contains a centrally located dark body and needlelike shape with a size of 18 to 23 nm. All coronavirus contains at least four structural proteins, of which the most significant is the S protein (spike), which is the carrier of enteral virulence). In the novel epidemics in China and the USA isolated viruses are genetically significantly different than already existing isolates. PED Viruses sensitive to ether chloroform, 2% NaOH, 1% formalin and rapidly loses infectivity at temperatures of 60◦C. The virus is transmitted by direct feco-oral transmission. Clinic symptoms occur 2 to 5 days after the entry of viruses in receptive population. Indirectly virus is transmitted through contaminated equipment, humans, etc. (38).

There are two types of PED virus: Type 1in fattening pigs and type 2 in pigs of all age categories. Virus damages the bowel villas and causes watery diarrhea as the basic clinical symptom. The disease is generally consisted of impossibility of nutrients uptake, rapidly loss of body weight and consequent dehydration. Piglets show strong diarrhea, rapid dehydration and possible high mortality, the most commonly occurring acute diarrhea without blood and vomiting. Mortality is usually low, but morbidity is high. When the virus is firstly introduced

into the herd after the incubation period of 2 to 4 days, 100% of the morbidity is reached in 5 to 10 days. In the pathomorphological finding, typical lesions thin and transparent walls of the duodenum, and the accumulation of large amounts of yellow fluid in the lumen of the intestine is observed. The gastrointestinal tract is most often filled with the contents, in suckling piglets - milk. Other internal organs have a normal appearance. Pathohistological findings of changed jejunitis and mild vacuolation of surface cells and subepithelial edema in cecum and colon (Jung et.al.2014).For material for laboratory diagnostics are samples of intestinal contents of dead animals, also possible are swabs (fecal nasal) and blood (blood serum) from live animals which is used in the serological diagnostics. ELISA tests and immunofluorescence often lack sensitivity and specificity. RT-PCRs using an M-based primer gives an adequate result. The use of immunohistochemical methods, as well as electron cystoscopy, is diagnostically relevant for differential diagnosis for the following diseases: - viral gastroenteritis (TGE, rotavirus) - bacterial gastroenteritis (*Clostridiumspp., E. coli, Salmonella spp., Brachyspira spp., Enterococcus durans, Lawsonia intracellularis*) - parasitic gastroenteritis (Coccidia, Cryptosporidium, Nematodes). The PED virus has no zoonotic potential, and the disease it causes is not OIE significant illness. The importance of PED virus is primarily in large pig breeding. The damage is attributed to: direct - dying and indirect, which include secondary bacterial infections and enlarged costs of feeding and the application of other biosecurity procedures on the farm (42). It should be noted that less damage is caused by the acute outbreak of the disease than the damage of attempts to eradicate viruses from the population. It has to be mentioned that in Europe the PED virus is not recognized as cause of major problems. However, because evolution of viruses we can also expect change in pathogenicity, and consequently larger economic damage to Europe's piglets.

Rota Virus Infection (RV)

Rotavirus (RV) infection is registered on all continents and is considered that virtually all adult pigs have RV antibodies. Viral replication is in the cytoplasm, and one of the characteristics of the virus is that it is a genetic material subject to the so-called reassorting. Tripsine lyses one of the capsid proteins (VP4) and increases the viral infectivity. High resistance of virus made them persist constantly in the objects and even in the "all in all out "system, so the excretion of the virus from the sows is considered significant for piglet infecting. As a result of oral infection, RV infects differentiated cell of the epithelium at the distal periphery of the intestinal reservoir of the small intestine.

Probably virus require activation by trypsin. For infection is particularly sensitive piglets during suckling and piglets up to three weeks of age. Diarrhea onset is known as the "spillage for the week". Diarrhea is the only and most significant symptom and it can be too mild to notice. In older categories, infections are unapparent. In the macroscopic examination of dead animals thin intestine wall is revealed with lumen full of yellow liquid content. The pathohistological finding

points to the atrophy of the villa, and their normal physiology function is returned for 9 days after infection. The disease prevalence is identical to the TGE (43).

Circoviral infection of pigs

Circoviral infections of pigs are popular in recent days causing great attention. Today, they present a great void in veterinary knowledge (25). Today classification describe: 1. Severe systemic infection of PCV2 (formerly known as PMWS) 2. PCV2 - associated pneumonia3. PCV2 - associated enteritis4. PCV2 - associated reproductive failure5. PCV2 - associated porcine dermatitis and nephropathy syndrome (PDNS). Circoviruses are named after ring DNA. Extremely small (17nm) and relatively resistant so they can stand at 70° C for 15 minutes. It is not known how much it spreads from one herd to the other, but as with other viruses, animal transport plays a significant role. It is excreted in faces and slime and it is spread by direct contact between and by aerosol. Since it is relatively resistant, the mechanical transmission is quite obvious (14, 15, 16, 41).

Porcine Dermatitis Nephropathy Syndrome (PDNS)

Porcine dermatitis nephropathy syndrome (PDNS) is new and economically significant disease that mostly affects pigs from 5 weeks to 5 months of age. It is seriously concerned that clinical signs and lesions resemble the pig's plague, and there is no knowledge of how it is transmitted and treated. The pathogenesis of the disease, to previous knowledge, as based on the role of hyperimune reactions with deposits of immunocomplexous on the walls of blood vessels, and it can progress to systemic necrotizing neuromusculitis. The most remarkable clinical symptom in the severely diseased piglets is dermatitis on chest, abdomen, femoral region, forelegs with appearance of purple-red swelling of various shapes and sizes. Pigs are depressed, febrile with reluctant movement and food consumption. In some cases, they show dyspnea. Animals recovering from the infection can have a slower growth (18, 22).

Reproductive Respiratory Disease (PRRS)

Reproductive Respiratory Syndrome (PRRS), a very contagious disease used to be originally called mysterious disease of pigs. Due to rapid spread to new areas and due to manifest clinical symptoms, it got different names in different countries like late abortion or blue ears disease. The mOIE and the International Minnesota Symposium have adopted the name porcine reproductive and respiratory syndrome (PRRS). The disease is caused by virus which is according to morphological material, method of replication and composition of proteins closest to the genus Arterivirus. Today, two standard isolates of the PRRS virus are known, one is European, another American. Different antigens between isolates from Europe and America are significant and it can be said that there are a European and an American type. PRRS has proven to be viral multisystem disease. Initially, it is manifested by viremia and after distribution of the virus to

many organs, where it's multiplied and cause pathological changes (pneumonia, vasculitis, myocarditis, lymphadenopathy, etc.).Clinical symptomatology depends on gestation period and on the age of the animals. Abortions may occur sporadically or massively, usually as late abortions, premature birth of avital piglets, and mummified fetuses. Pigs infected immediately after birth with the symptoms of severe dyspnea, conjunctivitis, edema of the eyelids, elevation of body temperature, trembling and slow coagulation of blood. In weaned piglets, PRRSV lead to fever, pneumonia, lethargy, lagging growth and a significant increase of mortality. In fattening pigs, boars and sows PRRSV cause transit hyperthermia and inapetence. Sometimes in boars, there can be loss of libido. Adult animals' seroconversion may be the only indicator that the PRRSV infection occurred (5, 40, 43, 44).

Parasitic infections

Intense farming properties are large agglomerations of animals in a limited area, which makes the risk of disease spreading increases along with the density of the agglomeration. The larger the number of animals in the smaller area, the possibility of spreading infections is higher since the conditions of accommodation and nutrition at a certain time are identical and equally favorable or negative for all individuals. Parasitic infections can be easily entered and disseminate in large number of individuals, with even mixed infections that greatly aggravate the diagnosis and treatment of diseased animals. Based on the examinations carried out on a large number of farms, we found that parasitic infections were not preclusive to any age group of pigs. Incidence and morbidity depend on the hygiene of keeping, preventive measures and the regularity of parasites control. In this case, the important moment in the emergence of infections is the first infection of the piglets through sows; these pigs with parasites go to the breeding and fattening (30, 33).

In post weaning period, the presence of protozoal infections is most commonly reported, so we have observed presence of *Balantidium coli* (95-100%), *Cryptosporidium parvum* (17-32%), *Eimeria perminuta* (27-31%), *E. debliecki* (3-24%), *E. politics* (4-9%) and *Isospora suis* (3-13%).

In older categories*, Ascaris suum* (39-41%), *Oesophagostomum dentatum* (6-8%), *Strongyloideus* (1-17%) and *Trichuris suis* (1-7%) were found. Mange caused by *Sarcoptes scabiei var. Suis* was found in 3-37% of pigs in this production period. *Ascariosis* is the most frequent parasitic disease of pigs in farm conditions and global distribution. It is a white-skinned nematode of a cylindrical body shape, whose male is 12-25 cm long and 3 mm wide and the female is 30-35 cm long and 5-6 mm wide. Developed larvae start the hepatopulmonary migratory phase when they are change stadiums four times (in the liver, lungs and intestines). After 8-10 days of infection, they are swallowed through the sputum and come into the intestine, where they become adult parasites and where the mating and eggs are placed. The clinical picture depends on the number of

parasite eggs intake, age and condition of the animals. The most expressed clinical picture is found in piglets 4-5 months old (in this group, significant mortality can be found) - in the form of cough, bronchopneumonia (due to larval migration of parasites), weight loss, poor progression and diarrhea. Pigs older than one year have developed immunity to this parasitosis, similar to analogous self-cure mechanism in ruminants (28).

Protozoa infections are most common in starting pigs, where there are many predisposing factors that affect the occurrence and outbreak of these diseases. Transition to the cage system has a stressful effect, which together with a change in the diet leads to the outbreak of these diseases, which can be tracked by significant diarrhea, weight loss, and death. The protozoal infections are often included in the syndrome of multiverse enteropathy of the piglets. The most important role in this is certainly the infection with *Isospora suis* and the zoonotic protozoa *Cryptosporidium spp.* (31, 32).

Pig mange is a parasitic disease caused by *Sarcoptes scabei* var.suis. It is a contagious disease, mostly chronic, characterized by itching and skin changes. Primary parasites' site is skin around the muzzle, eyes, and ears. If not treated it spreads on the neck, in the inner part of the legs, scrotum or vulva. If mange affect large area clinical signs may appear like inapetention and anemia with anxiety (34).

Conclusion

Today's industrial production of pigs is based on the implementation of biosecurity measures, as well as on solving environmental problems, which significantly burden the production. Good pig health is a requirement for good reproduction, that is, profitable production. The health condition depends on many factors, such as conditions of keeping, nursing, nutrition and implementation of preventive measures. Breeding diseases that are present in commercial farms can endanger the production of pigs in intensive care. They must be kept under control by prophylactic and therapeutic measures, as well as by strengthen the control of professional services. Flexible cooperation between farm owners with professional services, respecting and carrying out expert knowledge, applying a series of biotechnical measures and putting an emphasis on prevention of pig disease in order to promote good health of pigs, it is possible to improve production. Biosecurity, welfare, good manufacturing practices and risk analysis at critical control points are very important elements in intensive pig production. The planned application of biosecurity measures is crucial in protecting the health of pigs, and thus in the success of production.

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WELFARE ASSESSMENT OF BREEDING HORSES BY HEALTH AND BEHAVIORAL INDICATORS

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Summary

The welfare assessment of reproduction horses is important as it leads to the recognition of existing problems and their rapid remedial. This study assessed comparatively the welfare of two breeding horse categories: stallions and broodmares. The assessment included 27 stallions and 35 broodmares and it was based on health indicators (hair coat condition, hair quality in the mane/tail, body lesions, lower leg lesions, swollen tendons/joints, hoof horn quality, hoof walls' length, quality of horseshoes, gait, dyspnea, nasal discharge, diarrhea) and behavioral parameters (behavioral response towards humans). The data were analyzed using the SPSS statistical software. The value of minimal significance was considered at $P < 0.05$. The prevalence of stallions with dyspnea, tendon and joint swellings, abnormal gait and abnormal hoof horn quality was significantly ($P < 0.05$) higher than that of the broodmares. No significant difference was found in the behavioral response of the two categories of breeding horses, although the prevalence of indifference was higher in the breeding stallions in all of the three tests used. The welfare of the broodmares seems to be better than that of the breeding stallions, probably because of the different conditions they were kept in.

Keywords: breeding horses, health indicators, behavioral indicators, horses welfare

The welfare assessment of reproduction horses is important as it leads to the recognition of existing problems and their rapid remedial for improve the welfare of horses.

In the past 15 years the scientific research focused more than before on the welfare of working horses (1, 3, 10, 11) besides of those horses used by other means (5,14).

In this moment there is no widely accepted assessment protocol to evaluate the welfare of horses. During the past years a few researchers (1, 3, 4, 10, 11, 13) used animal-linked indicators (health and behavioural parameters), but also resource-based, indirect indicators, to assess considerably large horse populations. Recently the AWIN welfare assessment protocol for horses (2) was published, being based on the Welfare Quality® principles and criteria.

The studies about breeding horses' welfare are limited in the scientific literature (12).

The aim of this study was to assess comparatively the welfare of two breeding horse categories (stallions and broodmares) using health and behavioral parameters.

Materials and methods

The study was performed on a sample formed by 27 breeding stallions and 35 broodmares kept in a breeding farm in Transylvania. The stallions were housed tethered, in closed barns, with ground flooring, natural and artificial illumination, using mainly straw as bedding (and occasionally wood shaving) and having permanent access to water (automatic waterers). The cleaning of the barns and feeding of the horses was made manually, using manpower. The access for free exercise in this category was limited, because of the need to provide individual space for each animal (to avoid fighting) and human supervision. The mares were housed in the same type of barns but having daily access to free exercise, in groups, in the paddocks of the barns and also in the pasture (all day long in the warm season). In the day of arrival, half of the horses of the studfarm were assessed, their selection being done randomly.

The welfare assessment was made based on health and behavioral parameters by the methods described by Popescu and Diugan (10). Each horse was assessed by two experimented assessors. Within the health related parameters the followings were assessed: hair coat condition, hair quality in the mane/tail, body lesions, lower leg lesions, swollen tendons/joints, hoof horn quality, hoof walls' length, quality of horseshoes, gait, dyspnea, cough, nasal discharge and diarrhea. The general attitude of the breeding horses (apathetic or alert) was assessed and their reactions (aggressiveness, fear/avoidance, indifference, friendliness) were evaluated in response to the assessors' approach, walking besides and the attempt of touching the animal.

The data were analyzed using the SPSS statistical software. The prevalence of the assessed health and behavioral parameters was calculated in the stallions and broodmares. For comparison of data the Mann-Witney U test was used. The value of minimal significance was considered at $P < 0.05$.

Results and discussions

The prevalence of health parameters assessed in stallions and broodmares and the significance of differences between the two breeding horse categories is shown in Table 1.

In this study the most health problems were recorded in the stallions, probably because their tethered housing system. Similar results are reported by Sanmartín Sanchez et al. (12) in their study performed in Spain. The prevalence of stallions with tendon and joint swellings, dyspnea, abnormal gait and abnormal hoof horn quality was significantly ($P < 0.05$) higher than that of the broodmares.

Based on the investigation of interrelations between the welfare indicators assessed in Romanian working horses, Popescu et al. (9) states that the good quality of the haircoat seems to be a valuable parameter of improved welfare. In the present study the prevalence of normal haircoat was lower in the stallions than in the broodmares, even if the difference was not statistically significant (Table 1). The cause could be the lack of access to free exercise, including the natural behaviour of rolling which has a certain role in cleaning the skin and haircoat and maintaining a good health of these.

Table 1

The prevalence of health parameters assessed in stallions and broodmares and the significance of differences between two breeding horse categories

Parameter	Stallions (n= 27)	Broodmares (n=35)	P value
Hair coat condition (Abnormal)	14.81	5.71	0.112
Hair quality in the mane/tail (Abnormal)	11.11	17.14	0.507
Body lesions	14.81	8.57	0.422
Lower leg (foot) lesions	14.81	5.72	0.234
Swollen tendons/joints	22.22	0.00	0.002
Hoof horn quality (Abnormal)	29.63	0.00	0.001
Hoof walls too long or too short	25.93	14.29	0.144
Inadequate horseshoes	0.00	0.00	1.00
Gait (Abnormal)	59.26	5.71	0.001
Dyspnea (Present)	14.81	0.00	0.008
Cough (Present)	7.41	0.00	0.104
Nasal discharge (Present)	7.41	2.86	0.412
Diarrhoea (Present)	0.00	0.00	1.00

If P value is less than 0.05 the difference between stallions and broodmares is significant

The normal quality of hair in the mane and tail was more frequent in the stallions than in the mares. Even if the general aspect of the hairs is strongly related with the systemic health of the animal, the partial distruction of mane and tail could be a consequence of some management factors or behaviours.

The lesions, both on the bodies and on the lower legs of the horses, were more frequent in the breeding stallions than in the mares. The explanation lies in the fact that when physical contact was possible (accidental untethering in the barn, for example), the stallions were attacking each other, producing wounds and lesions by biting or by hitting each other with their legs and heads.

The swellings of tendons and/or joints were identifiend only in the stallions. Insufficient movement and tethered housing could represent important risk factors

for these problems. The prevalence of this parameter was lower than that reported in working horses (10,11).

The abnormal quality of hoof horn was observed only in the stallions, in which the frequency of too long hoof walls was higher than in the mares.

In this study the proportion of stallions that presented abnormal gait was significantly higher than that of the mares ($P < 0.05$). The main cause of this result is represented by tethered housing of the stallions. In the working horses higher prevalence of abnormal gait is reported (3, 11).

The indicators of the presence or absence of dyspnea, caughing and nasal discharge had the role of airway health assessment. As regards dyspnea, the differences were significant ($P < 0.05$) between the two categories of breeding horses. A respiratory system dysease affecting many horses is the recurrent airway obstruction, which involves both genetical and environmental factors (7). An important risk factor in occurence of this disease is the exposure to airborne allergens and specific aero-irritsnts, such as ammonia, molds and inhalable dust particles from the barn, especially in the absence of adequate ventilation. Of the horse categories assessed, the stallions spent the most time inside the barn, tethered.

It is remarcable the fact that in none of the breeding horse categories assessed had any signs of diarrhoea, probably because of regular deworming.

Table 2 shows the results of behavioural parameters assessed in stallions and broodmares and the significance of differences between two breeding horse categories.

No significant difference was found in the behavioral response towards humans of the two categories of breeding horses in none in the three tests performed. In this study, similar to the results of another recent research done in Romania, no apathetic mare was identified. These findings are in agreement with those obtained by Sanmartín Sanchez et al. (12). Aggressiveness was observed only in the mares in the test of the touch of the assessor.

The number of horses showing fear increased from one test to the other, both in the stallions and in the mares. The fear reaction of animals towards people is produced probably as effect of improper human attitude in the relations with the animal, leading to previous negative experiences. Recognizing this negative mental state is important, because fear and stress, both acute or chronic, can jeopardises the health and welfare of animals (6).

The frequency of indifference responses was higher in the stallions than in the mares, probably because of the housing management, tethered in closed barns.

Similar to other studies performed in Romania (8), aproximately half of the breeding horses, in both categories, showed friendly response to the human presence in the three behavioural tests. As general welfare considerents, the friendly reaction of horses to humans is the most wanted behavioual response.

Table 2

The prevalence of behavioural parameters assessed in stallions and broodmares and the significance of differences between two breeding horse categories

Parameter	Stallions (n= 27)	Broodmares (n=35)	P value
General alertness			
Apathetic/depressed	3.70	0.00	0.255
Alert	96.30	100.00	0.231
Response to the approach of the assessor			
Aggressiveness	0.00	0.00	
Fear/avoidance	22.22	22.86	0.118
Indifference	29.63	22.86	0.136
Friendliness	48.15	54.28	0. 105
Response to the assessor walking besides			
Aggressiveness	0.00	0.00	1.00
Fear/avoidance	29.63	28.57	0.725
Indifference	18.52	14.29	0. 681
Friendliness	51.85	57.14	0.643
Response to the touch of the assessor			
Aggressiveness	0.00	2.86	0.537
Fear/avoidance	37.03	42.86	0.421
Indifference	14.82	8.57	0.459
Friendliness	48.15	45.71	0.602

If P value is less than 0.05 the difference between LHS and THS is significant

Conclusions

The welfare of the broodmares seems to be better than that of the breeding stallions, probably because of the different conditions they were kept in.

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THE USE OF IMMUNOHISTOCHEMICAL TECHNIQUE AS A ROUTINE METHOD FOR THE DIAGNOSIS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME 1

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Summary

Porcine reproductive and respiratory syndrome (PRRS) was diagnosed, in 1987, in the USA and Canada and in Europe was diagnosed for the first time in November 1990 in Germany. For detection of the PRRS (nucleocapsid) virus antigens present in infected cells, a diagnostic kit containing a conjugate of specific immunoglobulin coupled to peroxidase was used. For this purpose, inguinal lymph nodes were taken from swine youth bodies after weaning, who had macroscopic lesions specific to PRRS syndrome. Immunohistochemically, a total of 28 inguinal lymph nodes were collected from 25 corpses . From each lymph node were taken samples of approximately parallelepiped form which were intended for the working protocol which comprised 3 parts. In the examined sections the brown color, present in the cell cytoplasm of germinating centers of the medulla zone, was highlighted. This aspect is considered to be expression of PRRSV1, a positive image for the presence of viral antigens in lymphocyte cytoplasm of the lymphocyte crown. Also by this technique were areas of necrosis and depletion of these centers. The characteristic aspect provided by the IHC technique used was present in the sections taken from 23 lymph nodes, and in two lymph nodes this expression (brown color) was absent, the results being considered negative.

Keywords: PRRS, Immunohistochemical, Inguinal lymphonode, brown color

Porcine reproductive and respiratory syndrome (PRRS) was diagnosed, in 1987, in the USA and Canada and in Europe was diagnosed for the first time in November 1990 in Germany (7).

The disease has spread very rapidly worldwide, and is currently developing as endemic outbreaks in swine farms. It produces significant economic loses through mortality, abortion, infertility, immunosuppression followed by secondary bacterial infections, low conversion of feed and non-weight gain, diagnosis and epidemic surveillance costs, and costs of non-specific, specific and control prophylaxis. In view of these aspects, PRRS syndrome is included in TAHC, the

Suidae (15) section, Chapter 15.3, under the name of Infection with porcine reproductive and respiratory syndrome virus (8).

Diagnosis of the disease involves the detection of the etiological agent by: virological examination, electronic microscopy - negative staining, direct detection of viral antigens (immunohistochemistry, indirect fluorescence), nucleic acid detection by RT-PCR, in situ hybridization and loop mediated isothermal amplification; detecting antibodies by: IFD, ELISA, Virus neutralization (VN) and IPA (7,8).

The immunohistochemical test reveals, in the cytoplasm of infected cells viral antigens and nucleocapsid, by means of monoclonal antibodies with which they form antigen-antibody complexes, visualized by microscope by means of conjugates with different composition. Immunohistochemical technique has been used more extensively in experimental research and has been less commonly used as a routine method in the diagnosis of this disease because it requires diagnostic kits, technical equipment and staff with experience in interpreting the results (7,8).

Research has been done to use the immunohistochemical technique in the diagnosis of PRRS syndrome with the Indirect immunoperoxidase assay kit provided by BIO-X Diagnostics.

Materials and Methods

For detection of the PRRS (nucleocapsid) virus antigens present in infected cells, a diagnostic kit containing a conjugate of specific immunoglobulin coupled to peroxidase was used. For this purpose, inguinal lymph nodes were taken from swine youth bodies after weaning, who had macroscopic lesions specific to PRRS syndrome. Immunohistochemically, a total of 28 inguinal lymph nodes were collected from 25 corpses.

From each lymph node were taken samples of approximately parallelepiped form which were intended for the working protocol which comprised 3 parts.

In Part I, each sample was fixed in 4% paraformaldehyde for 24 hours, after which it was washed in tap water and held in: 50% alcohol (1 hour), 70% alcohol (1 hour) , alcohol: 95% (1 hour), 100% alcohol (1 hour), alcohol: toluene (1 hour), alcohol: 1: 1 toluene (1 hour) 1 hour). The samples were placed in paraffin I enclosure boxes and kept in a thermostat at 60 ℃ to 2 o'clock a day and paraffin II kept in a thermostat at 60 ℃ for one hour. The paraffin used had the following composition: 100g paraffin + 5g wax.

Parts 2 and 3 of the technique used were performed according to the Novolink Polymer Detection System immunohistochemical protocol.

In the 2 part, the blocks were cut on microtome and the sections were deposited on glass blades, followed by the following steps: dewaxing the blades with toluene (2 baths for 15 minutes each), rehydration of the sections with ethanol (100% 5 min, 96% -5min, 70% - 5 min), washing the blades with distilled water and removing excess water, neutralizing endogenous peroxidase with PEROXIDASE

BLOCK for 10 minutes, washing with TBS 1 (2 baths for 5 minutes) incubating with PROTEIN BLOCK (10 minutes), washing with TBS 1 (2 baths of 5 minutes).

Following these steps, the primary antibody consisted of the immunoperoxidase conjugate in the dilution of 1: 100 was added to the blades, after which the lamellae were kept in water trays in the refrigerator up to next day.

In the third part after removal from the refrigerator, the lamellae were subjected to the following steps: washing with TBS 1 (2 baths for 5 minutes), incubating with POST PRIMARY (30 minutes), washing with TBS (2 baths of 5 minutes) , incubation with NOVOLINK POLYMER (for 30 minutes), TBS 1 wash (2 bath for 5 minutes), treatment of blades with DAB WORKING SOLUTION (5 minutes), washing with H2O2, contraction with hematoxylin (40 seconds), washing with water distilled (2 baths for 5 minutes) and washing the blades with: UNYHOL, UNYHOL PLUS and BIOCLEAR (9,10).

Finally, the blades were dried, the lamellae were fixed and examined under a microscope.

Results and Discussions

Necropsy examinations performed on swine youth corps after weaning, revealed macroscopic lesions characteristic of PRRS syndrome in the lungs and lymph nodes. From a number of 25 corpses, with lesions specific to this disease, inguinal lymph nodes were shown to have bloody and haemorrhagic lymph nodes (Fig. 1).

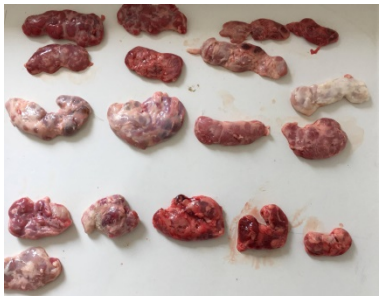

Fig. 1. Inguinal lymph nodes with cataraural and haemorrhagic lymph nodes

Samples of lymph nodes were processed according to the presented methodology, after which they were examined under a microscope. Particular attention has been paid to the 4% paraformaldehyde fixation step because correct fixation of samples protects epitopes (viral antigens) and improper attachment can destroy or inactivate epitopes resulting in false negative reactions.

The working method used allowed the fixation of the primary antibodies coupled to the peroxidase present in the viral nucleocapsid kit used, and

subsequently the antigen-antibody-peroxidase complexes were visualized with the help of the secondary antibodies and, by adding 3,3'-diaminobenzidine, which reacts with the peroxidase resulting in a dark brown granular color indicating the presence of viral antigens in the cell cytoplasm.

In the examined sections the brown color, present in the cell cytoplasm of germinating centers of the medulla zone, was highlighted. This aspect is considered to be expression of PRRSV1, a positive image for the presence of viral antigens in lymphocyte cytoplasm of the lymphocyte crown. Also by this technique were areas of necrosis and depletion of these centers (Fig. 2, 3).

Fig. 2. Lymphoid section: necrosis and germination center depletion

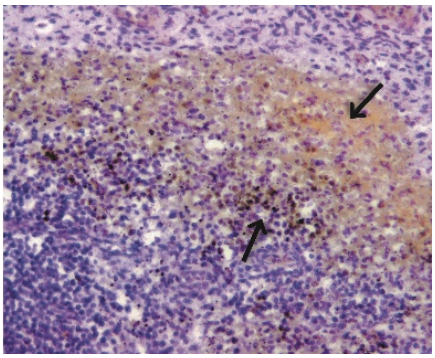

Fig. 3. Section by lymph node: cells with brown granular cytoplasm

The characteristic aspect provided by the IHC technique used was present in the sections taken from 23 lymph nodes, and in two lymph nodes this expression (brown color) was absent, the results being considered negative. By the techniques

recommended (RT-rPCR) by GILBERT et al. (1997), cited by FLUERASU L. et al (2016), the virus was detected in all examined samples (1).

The results obtained showed a good correlation between the two diagnostic techniques, even if the results obtained by the IHC technique were negative in two samples. The results obtained using the IHC technique are similar to the results in the literature on the use of this method both in PRRS diagnosis and in various researches.

HALBU R P.G. et al., in 1995, carried out research on the diagnosis of PRRS with immunohistochemical technique using monoclonal antibodies as primary antibodies and revealed the presence of viral antigens (nucleocapsid) in lymphoid and spleen cells with a higher frequency than in other tissues. Also, areas of follicular necrosis in lymph nodes have been highlighted (2).

THANAWONGNUWECH, R. et al., in 1997, used, for the purpose of immunohistochemical diagnosis to highlight the presence of PRRSV antigens in various tissues, and found that most positive results were in lymphocytes and macrophages in lymph nodes to which it was highlighted a brown granular staining in the cytoplasm. Frequent follicular lymphocytic necrosis has also been highlighted (5).

VAN ALSTINE W.G. et al., in 2002, investigated the use of the IHC technique as a method of diagnosing PRRS syndrome. The authors found that sampling, sample transport, fixation, formulas, paraffin inclusion, as well as other stages of the technique may influence the results more accurately, contribute to the diminution of positive results and the increase in false negative results (6).

HAN K. et al., In 2012, used 3 laboratory diagnostic techniques for PRRS diagnosis. The immunohistochemical technique revealed viral antigens of types 1 and 2 in the cell cytoplasm of various organs, and by in situ hybridization and the Nested RT-PCR technique detected the viral genome. The authors found that the best correlation was between the results of the immunohistochemical technique and the in situ hybridization technique (3).

MANZANO LAVINA G. in 2017, conducted an extensive study on the diagnosis of PRRS in swine using histological and immunohistochemical technique. With the help of histological technique, it has revealed disease-specific lesions in lymph nodes, lungs, spleen, cord and kidneys, and the results confirmed that specific lesions have the highest frequency in lymph nodes and lungs. Through the IHC technique, viral antigens coupled to antibodies in the form of brown complexes (positive reactions) were detected in the cytoplasm of the cells in the organs. The authors stated that most positive reactions were found in lymph nodes and lungs, and negative results may not actually indicate the absence of the virus, as a result of inappropriate fixations and manipulations of the samples taken (4).

Conclusions

IHC technique revealed brown-gray viral antigens in cell cytoplasm of lymph node germination centers;

In the variant used IHC technique can be used as a method in the routine diagnosis of PRRS syndrome;

The obtained results were compared with the RT-rPCR technique, with a good correlation between the two methods.

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USING AEROBIC PLATE COUNT AS MICROBIAL INDICATOR OF URBAN ENVIRONMENTAL HYGIENE

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Summary

The aim of this paper was to assess the development of germs on the surface of the secondary pre-collecting bins (the household waste bins serving the blocks of flats), during summer, at different temperature values of the air and the bins.

There were collected 172 sanitation samples from the surfaces of the handles of the waste bins, component parts that are most likely to be touched when the garbage bags are taken to the bins. There have been done successive dilutions from the native samples and subsequently, inoculations into nutrient agar, in order to determine the aerobic plate count (APC), concurrently with the registration of the air and household waste bins temperatures.

Following the results obtained, the largest germ load was found on the bins that had temperature values between 22 and 37˚C, temperature considered optimal for the growth of mesophilic flora; on rainy weather, due to the lower temperature (<22˚C) and the washing effect of the rain, the germ load decreased, suggesting that by performing regular cleaning, with appropriate solutions, there would be a greater decrease in the number of germs; despite the high temperatures of the bins (over 45˚C), the aerobic plate count still remained raised.

Keywords: APC, urban environmental hygiene

The new legislative provisions on waste regime and the Order of Health Minister no. 119/2014, for the aproval of Hygiene and public health standards regarding the population's living environment, assigns to both citizens and local public authorities, certain obligations regarding the selective collection of household waste, in order to reduce environmental pollution and to comply with certain hygiene rules so as not to endanger public health (4, 5).

Some studies focused on showing the extent of microbial contamination of the various public surfaces (2) and inside the home (3), by determining the total germ load or by trying to put in evidence the presence of specific pathogens. However, no reference values of the total germ load on these surfaces have yet been established, values that could be correlated with a certain degree of cleanliness. For example, "BC Centre of Disease Control", an institution which provides health promotion and prevention services, analytical and policy support to government and health authorities, and diagnostic and treatment services to reduce communicable and chronic disease, preventable injury and environmental health risks, suggests that for samples taken from any surface relevant for

investigation (in this category are included door knobs, door handles and any handle) from public places like daycares, restaurants, community care facilities, schools, petting zoos, tatoo and body piercing or nail and manicure establishments, can be considered the following guideline for interpretation (for 25 cm^2 sampling area): clean – less than 5 CFU/cm^2, contaminated – between 5 to 10 CFU/cm^2, very contaminated – greater than 10 CFU/cm^2 (6).

The previsions of Order no. 119/2014, addressed to citizens, include: separate collecting of recyclable waste from the waste that includes food scraps, the necessity of using trash bags and maintaining the cleanliness of the household waste bins by periodical washing. The purpose of these measures is to prevent the development of potentially pathogenic germs inside and on the surface of these bins, to avoid attracting vectors (birds, rodents, insects) and to prevent waste pollution of the area where these bins are placed.

The aim of this paper was to assess the development of germs on the surface of the secondary pre-collecting bins (the household waste bins serving the blocks of flats), during summer, at different temperature values of the air and bins, given that most of these waste collecting places did not meet the location requirements set out in the Order of Health Minister no. 119/2014, Chapter I - Hygiene Standards for Living Areas, Art. 4 (a) (1).

Materials and methods

There were collected 172 sanitation samples from the same number of waste collecting places (a collecting place consisting in one or two plastic household waste bins). Sampling was performed from the surface of the handles of the waste bins, component parts that are most likely to be touched when the garbage bags are taken to the bins.

Sampling was performed in different areas from city of Timisoara, in the summer of 2016. The samples were collected at different times of the day, at different air temperatures (25˚C, 28˚C, 32˚C), under different conditions of air humidity (presence/absence of rainfall), from waste bins placed in the shade or directly exposed to the sunlight.

The bins temperature was measured using an infrared thermometer with a measuring range between -50 and +380˚C. Samples were grouped into four categories, depending on the registered temperature:

- $T^{\circ}C_{bin} < 22^{\circ}C$ – 80 samples,
- $T^{\circ}C_{bin}$ 22-37˚C – 40 samples,
- $T^{\circ}C_{bin}$ 37-45˚C – 24 samples,
- $T^{\circ}C_{bin} > 45^{\circ}C$ – 28 samples.

There were performed successive decimal dilutions (10^{-1}, 10^{-2}) from the native sample. Inoculations were made by embedding into nutrient agar, in two Petri plates per dilution. The plates were incubated for 24 hours at 37°C, and after that, it was quantified the aerobic plate count (APC).

Results and discussions

After colony counting, we have obtained the results from Table 1, graphically represented in fig. 1.

Table 1

APC values

Bin temperature	APC (CFU/ml)
T˚C$_{bin}$< 22˚C, rainfall	1170
T˚C$_{bin}$ 22-37˚C	2451
T˚C$_{bin}$ 37-45˚C	1958
T˚C$_{bin}$> 45˚C	1431

Analyzing the data obtained, it appears that there is a linear correlation between temperature and the number of CFU obtained. Thus, if at low temperature (22˚C) and wet weather conditions, which acted by washing the bins, we obtained the least number of CFU/ml (1170), at temperature values appropriate for the development of mezophilic germs (22-37˚C), there has been obtained the highest number of CFU/ml (2451).

Also, the fact that even at temperatures values between 37 and 45˚C, has developed a large number of germs (1958 CFU/ml), is explained by the existence of the suitable growth substrate and by the temperature, which was still favorable for the multiplication of mesophilic flora. At temperature values higher than 45˚C, we found both mesophilic and termophilic flora. Although numerically there were less CFU/ml in comparison with the microbial loads found at other temperature ranges, the microbial load was still considered high.

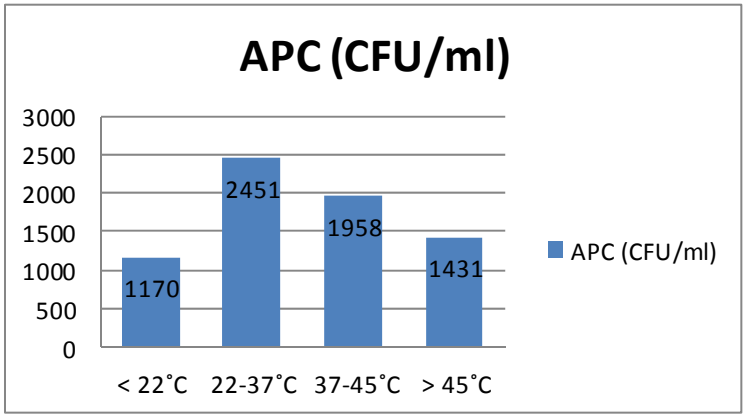

Fig.1. CFU dynamics of APC

Conclusions

It has been found that most germs have developed on the surfaces of the bins with temperatures ranged from 22 to 37°C, the optimal temperature for the growth of mesophilic flora.

On rainy weather, due to the temperature drop (<22˚C) and by the washing effect of the rainfall, the multiplication of the germs was slowed down, suggesting that by regular cleaning, using appropriate solutions, it could result in a further decrease in the number of germs.

At temperature values higher than 45˚C, although numerically there were less CFU/ml in comparison with the microbial loads found at other temperature ranges, the microbial load was still considered high.

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RISK FACTORS FOR DIARRHEA IN CALVES UNDER ONE MONTH OF AGE

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Summary

The study was conducted in four regions from Algeria and involved 816 veal belonging to 53 randomly selected beef herds in order to determine the prevalence of diarrhea and mortality and the association of a number of suspected risk factors among calves less than 30 days of age. A questionnaire has been provided to report diarrhea onset, mortality and risk factor correlated with. The morbidity rate was of 29.17 % and the death rate of 11.76 %. Using logistic regression analysis, it appeared the significant risk factors were age, season, nursing area, multiparity and navel disinfection. However, maternal nutrition and sex of newborn are likely to not be risk factors for diarrhea onset.

Keywords: Algeria, cattle, digestive trouble; newborn, study.

Calves production is an essential outlet for breeding as it provides consumers with meat and industrial dairy products. Starting at the crucial birth, the calf is subjected to attacks from the outside environment where numerous pathogenic agents can colonize the respiratory and digestive systems leading to several diseases (1; 2).

Neonatal diarrheas occupy an important place among the young calf diseases with 60 to 80 % of total affections in newborn (3); this is also one of the most murderous diseases (4). This mortality is determined by various factors including economic losses due to therapeutic expenses often ineffective, long convalescence period, costs due to stunting and delayed calving (5; 6).

The disease severity and outcome result also from interactions between intrinsic factors specific to the animal (calf age, time of colostrum intake, its quantity, and quality, stress) and environmental factors (litter renewal, overcrowding, hygiene, individual stall).

Some elements (age at the diarrhea onset, dehydration, mortality, appearance of feces, general signs associated) suggest a cause rather than another but there are no peculiar signs allowing knowing exactly the responsible agent.

Indeed, knowledge of herd management and environmental factors would improve the ability to control and prevent diarrhea on herd health and productivity (7).

Therefore, the main objective of the present study was to determine the prevalence of diarrhea in veal calves less than one-month-old in four regions in Algeria and to analyze the association between diarrhea rate with age, sex, and other factors.

Material and Methods

A descriptive survey was conducted during two years starting October 2011, on 816 veal no more than one-month-old.

Fifty-three (53) randomized dairy farms from four Algerian regions: East, Center, West, and South were chosen for this study.

All cattle farms were Friesian breeds was carried out in four Algeria regions. The size of the dairy cattle herd varied from 3 to 21 (mean: 10 ± 4) according to the herd

The identification of the diarrhea affections was based mainly on symptomatic statements (watery stools).

Data on potential risk factors were obtained using a questionnaire comprising: practices of breeding (livestock housing, hygiene measures, availability of calving area and individual pens, maternal nutrition/vaccination, parity, dry cow stage, colostrum intake, navel disinfection and the cattle sex; onset of diarrhea (1st, 2nd, 3rd or 4th week) and the season (autumn, winter, spring or summer).

Definitions:

Morbidity rate: the number of diarrheic veal divided by the number of total veal.

Cases fatality rate = the number of death divided by total number of diarrheic calves.

Death rate = the number of deaths divided by total calves.

The descriptive and statistical analyses were made by means of two Microsoft Excel Service 2007 software and SAS Version 8.2 (Cary, n.c).

Results and discussion

Among studied livestock, two hundred and thirty-eight (238) calves were classified as cases (diarrheic). Of these, 107 had diarrhea during the first week, 89 the second week, 31 the third week and finally eleven the fourth week (Table 1). Diarrhea was mainly recorded during the first week (13.12 %) and then decrease gradually.

In calves, neonatal gastroenteritis has multiple etiologies; they remain a complex and multifactorial pathological entity due to several causative agents: *Escherichia* coli, *Rotaviruses*, Coronaviruses and *Cryptosporidium* (8, 9, 10). It is

one of the most common diseases in young animals, causing huge economic and productivity losses to the bovine industry worldwide (11). However, *Cryptosporidium* was the most frequent pathogens that induces diarrhea at calves, mostly associated with coronaviruses (12, 13).

Table 1

Distribution of diarrheas and mortality by age

	Week 1	Week 2	Week 3	Week 4	Total
Diarrheic calves	107	89	31	11	238
Morbidity rate (%)	13.811	10.90	3.80	1.35	29.17
Deaths	61	35	00	00	96
Cases fatality rate (%)	25.63	14.7	00	00	40.34
Death rate (%)	7.47	4.29	00	00	11.76

The overall prevalence observed (29.17%) is close to the one reported by Schumann et al. (13) with 21.98%.

However, in Algeria Ouchene et al. (14) reported the lowest prevalence with 15 % while Boussenna and Sfaksil (15) found a higher one (64%) in the Eastern region. The difference in morbidity seems correlated to the hygiene respect and bred management.

Most of the observed cases (82.35 %) appeared during the first two weeks; in line with other research (14; 16; 17), reporting gastrointestinal disorders during the first 15 days of life, essentially during the first week.

Deaths were recorded only during the first and second week affecting more than 40 % of diarrheic animals.

Highly significant differences were found between seasons ($p = 0.0095$). The diarrhea was maximal in spring (8.95 %) and minimal in summer (4.66 %) (Table 2).

Table 2

Distribution of diarrheas according to the season

	Winter	Springs	Summer	Autumn	Total
Diarrhea	65	73	38	62	238
Morbidity rate	7.96	8.95	4.66	7.6	29.17

Breeding practices: Colostrum intake was subjectively judged on a description by the farmer as to how he evaluated the intake of colostrum for each calf.

The risk of diarrhea is significantly increased by the absence of calving room, the absence of individual pen, poor hygiene, multiparity and mastitis (Table 3). A number of risk factors for neonatal calf diarrhea were identified in this study.

Indeed, certain parameters as calving room, individual pens, and cow parity have a significant effect ($p<0.05$), similarly to other works (18; 19; 20).

In that connection, livestock housing maintenance and hygiene are important to reduce the risk of disease transmission (21).

Table 3

Risk factors frequencies

Variable	Category	Frequency	%	P
Calving room	Yes	47	19.7	
	No	191	80.25	0.0070
Individual pens	Yes	51	21.43	
	No	187	78.57	0.0002
Stable hygiène	Good	31	13.03	
	average	149	62.61	0.0000
	Poor	58	24.37	
Maternal nutrition at the end of gestation	balanced	128	53.78	
	Unbalanced	110	46.22	0.0073
Dry cow stage	7th month	71	29.83	
	8th month	133	55.88	0.0000
	9th month	34	14.29	
Cow parity	Primiparous	60	25,21%	
	Multiparous	178	74.79	0.026
Mastitis	Yes	102	42.86	
	No	136	57.14	0.000
Prepartum vaccination (against rotavirus, coronavirus,ECV	Yes	10	4,20	
	No	228	95.80	0.014
Deworming	Yes	231	97.06	
	No	7	2.94	0.0017
Sex of the newborn	Male	125	52.52	
	Female	113	47.48	0.0000

It is interesting to note that, either maternal nutrition and sex at the birth are not risk factors.

The no navel disinfection in 60.08 % of calving, was significantly linked with diarrhea onset and time of first colostrum was during the first 6 hours in 76.47 % of calving and no probe was used to feed the veal (Table 4).

Another factor that appears to strongly influence the risk of diarrhea is the time of the first take, which is very important for the passive transfer of immunoglobulin (22; 23;24). The majority of farmers (76.47%) reported that calves received colostrum within 6 hours after birth; delays in the colostrum intake are due to dystocic calving (or caesarean) therefore the mother cannot breastfeed its

product and/or the calf cannot reach the mother teats also nocturnal deliveries especially when the mother is tied.

Table 4

Association between diarrhea onset and calve nutrition/treatment

Variable	Category	Frequency	%	P
Navel Disinfection	Yes	95	39.92	0.0026
	No	143	60.08	
Time of first colostrum	< 6h	182	76.47	0.0014
	> 6h	43	18.07	
	12h later	13	5.46	
Administration mode	Suckling	225	94.54	0.0289
	Feeding Bottle	13	5.46	
	Probe	0	00	

Maternal feeding was balanced in 53.78 % of calving cows, however, this factor was not significantly associated with diarrheas (p=0.71) similarly to another work (15).

In almost all visited farms, the calves suckled their dam, which could be a risk factor when associated with a poor passive immunoglobulin transfer (25). In our study, these factors had a significant effect on the occurrence of diarrhea.

Mastitis was significantly associated with diarrhea, which may be due to poor hygiene (20); a parameter often neglected by breeders.

Maternal feeding and drying can decrease significantly (P <0.05) occurrence of diarrhea as reported by Bendali et al. (16) due to proteins intake and subsequent antibody production.

Vaccination was not performed for more than 95.8 % of calving cows, which significantly increase diarrhea cases, in agreement with experimental evidence (17; 26). Gonzalez et al. (27) confirmed vaccination against enteropathogens was associated with decreased odds of liquid feces.

Males newborn cattle were more subjects to diarrhea than females similarly to another work (28), but further studies are needed to assess this difference.

Conclusions

The morbidity and fatality were very high due to several factors such as management (calving area, individual boxes), hygiene, multiparity, vaccination, deworming and mastitis confirming their multifactorial nature.

Although these factors favoring the onset and rapid progression of diarrhea should be taken into account, the management including hygiene improvement is essential keys to improve the treatment and especially the prevention of this disease.

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EFFECTIVE METHODS TO DETECT FELINE CORONAVIRUSES INFECTIONS

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Summary

Feline coronaviruses can be detected in feces, diseased tissues and fluids using various methods. The difficulties for diagnostic arise sometimes from the lack of specific clinical signs and pathognomonic abnormalities. **I**n this study we compared the results of various tests (immunofluorescence, ELISA, RT-PCR and immunohistochemistry) used to identify the feline coronavirus in clinical samples (feces, ascitic fluid, blood, kidney, intestine) harvested from 20 domestic and wild cats (10 healthy felines and 10 felines presenting clinical signs of a wet form of feline infectious peritonitis). Feline coronavirus was detected in all 10 samples of feces from the healthy cats and all ascitic fluid, 4 intestine and 2 kidney samples from the felines with clinical signs. Positive coronavirus antibodies titers are misleading because only show evidence of exposure to FCoV and are not specific for FIP or EFCV In conclusion, direct immunofluorescence assay is an alternative method to detect feline coronavirus in clinical samples, much faster and less expensive. Molecular biology offers instead the possibility to analyse the coronavirus strains circulating in Romania.

Keywords: FCoV, RT-PCR, immunohistochemistry, ELISA, FIP

Feline coronavirosis are infectious diseases produced by positive-stranded RNA viruses, *Alphacoronavirus* 1 species, *Alphacoronavirus* genus, *Coronaviridae* family, *Nidovirales* Order. The *Alphacoronavirus* 1 group, except feline coronavirus (FCoV), also includes the canine coronavirus (CCoV) and the swine transmissible gastroenteritis coronavirus (TGEV) (1, 4, 19). Feline coronavirus can be found in two different forms: the feline enteric coronavirus (FECV) with tropism for the enterocytes, inducing transient anorexia, weight loss or mild diarrhoea and the feline infectious peritonitis virus (FIPV) that causes the lethal systemic disease (6,14). Feline coronavirus is shed in feces by healthy domestic and wild cats and transmitted by the fecal-oral route to other receptor animals (5, 21, 22). Higher risks present the environments with multiple cats. It is now generally accepted that FIPV evolves from FECV through mutations in persistently infected animals. The amino acid substitutions M1058L and S1060A in the spike protein of feline coronavirus (FCoV) have been postulated to be responsible for the development of the pathogenic feline infectious peritonitis virus (FIPV), which causes feline

infectious peritonitis (FIP), a disease with no known cure, just symptomatic and palliative treatment (2, 4, 10, 13, 16, 17).

Generally, FCoV can be identified in various pathological samples such as saliva, feces, blood, pleural, ascites fluids, cerebrospinal fluid, organs, etc. of the infected cats. Therefore, cat-to-cat contact and exposure to feces in litter boxes is the most common way of infection. Contaminated food or water dishes, bedding, and personal clothing may also serve as sources of infection (7, 8, 11).

If the infection with the EFCV can evolve unnoticed, in case of FIP, there are two forms of the disease. The most obvious is the wet form, when vasculitis produced by immune complexes, complement and cytokines is more or less observed, depending on the amount of accumulated liquid and localization. The result is the appearance of the effusions in the abdominal (ascites fluid) or thoracic cavity (pleural, pericardial). All those fluids can be used to identify the FIPV, because, by mutation, the enterocyte tropism is abrogated and the monocyte/macrophages tropism develops (2,10). In the dry form of the disease the clinical signs are less characteristic, except fever, anorexia, weight loss, lethargy, uveitis, epiletiform seizures, nystagmus and paresis. At the necropsy, microgranulomas can be observed in various organs (7, 8).

Due to the lack of a non-specific symptom for feline coronaviruses infections, in this study we tested and compared several techniques (direct and indirect immunofluorescence, ELISA, RT-PCR, immunohistochemistry) generally used to identify coronaviruses and specific antibodies in different specimens harvested from alive animals and from corpses.

Materials and methods

The evaluation was performed on 10 healthy domestic cats, 9 domestic cats and 1 tiger presenting clinical signs of a wet form of feline infectious peritonitis. From the healthy animals, blood (n=10) and feces (n=10) samples were collected. From the felines with clinical signs of FIP, ascites fluid (n=10), feces (n=10), blood (n=10) and various organs (liver, kidney, lymph node, intestine) samples were harvested. Depending on the specimen, one or more diagnostic methods were used. The diagnostic on FCoV infection seems to be easy, but difficulties arise because the symptoms are common to many other diseases, and the tests that can be applied have a more or less diagnostic value.

Blood samples and ascites fluid were collected on EDTA (ethylene diamine tetraacetic acid) tubes (for the RNA extraction) and, in case of blood, tubes with clot activator factor for the serum. Organ samples were collected after necropsy was performed. Feces samples were suspended 1:1 (vol/vol) in phosphate-buffered saline (PBS) and homogenized by vigorous vortexing. Insoluble components were pelleted for 10 min at 13000 rpm (11). The supernatant fraction (that contain the virus), the organ samples, ascetic fluid were stored at -80°C prior to RNA extraction. Serum samples were stored on -20^{0}C.

To identify the FCov, RT-PCR, immunohistochemistry and direct immunofluorescence were used.

RT-PCR (Revers Transcriptase Polymerase Chain Reaction)

Viral RNA was extracted from clinical samples (blood, ascites fluid, feces) using QIAamp Viral RNA Mini kit, Qiagen, and from organs using Fluka RNA isolation kit, according to the manufacturer‘s protocole. The pellet was resuspended in 80 µl of RNase-free water and stored at -80°C prior to analysis by RT-PCR. The most well conserved region of virus genome, common in all coronaviruses from group I, was amplified usind a pair of primers (P205 GGCAACCCGATGTTTAAAACTGG 1–23 Sense, P211 CACTAGATCCAGACGTTAGCTC 213–192 Antisense) that amplified a fragment of 223 base pairs (bp).

The amplification was performed in 40 cycles (4,30 hours): reverse transcription (30 minutes at 50°C), activation of the polymerase (15 minutes at 95°C), denaturation of the DNA strand (1 minute 94°C), primers annealing to DNA (1 minute at 48°C), polymerization (1 minute at 72°C), final extension (10 minutes at 72°C). Amplified DNA fragments were separated by electrophoresis in 2% agarose gel, with GelPilot DNA Loading Dye, at 200mA, 80V for one hour. DNA Molecular Weight Markers GelPilot was used as molecular weight marker. The results were analysed using UV light (BIORAD DOC).

Direct immunofluorescence assay (DIF)

Ascites fluid was centrifugated for 10 min at 1000 rpm. The supernatant was discard and the pellet was displayed on a glass slide, the same as the feces and organ samples. The smears were fixed with ethanol 96% for 10 min, washed for 10 min in PBS 1x, on magnetic stirer. The highlighting of the coronavirus was realized with the use of primary (polyclonal antibodies specific to coronavirus, obtained from a positive ascites fluid) and secondary antibodies (fluorescein isothiocyanate-conjugated goat anti-feline antiserum 1/50) (Sigma) (350 µl each), followed by incubation for 60 min at 37°C. Labeled antibodies – coronavirus complexes were observed with IX51 Olympus inverted microscope.

Immunohistochemistry (IHC)

The organ samples were fixed, trimmed, embedded in paraffin. The 4 µm thickness sections were de-waxed and epitopes revealing was realized using heat in 10 mmol citric acid buffer (pH 6) for 10 minutes at 95°C in a microwave oven. The slides were left at room temperature for 20 min and washed twice in PBS (pH 7.5) for 5 min. Tissue sections were incubated with goat blocking serum, then with primary antibodies (Pierce monoclonal mouse anti-coronavirus antibodies, Thermo scientific) diluted 1:100 at the room temperature in a humid chamber for one hour. After being washed with PBS, slides were incubated with the secondary antibody (HRP Goat anti Mouse IgG), for 1 hour, in a humid chamber, at 4°C. After washing with PBS, the slides were incubated with ABC Kit for 30 min in a humid chamber, then washed with PBS, incubated with DAB substrate for 5 min, counter-stained with Harris haematoxylin, clarified in xylene and mounted. Also, the slides with the

sectioned tissue samples were used for the fluorescent antibody technique, using the mentioned protocole.

To highlight the specific feline coronavirus antibodies indirect immunofluorescence and ELISA method were used.

Indirect immunofluorescence assay (IFA)

This assay was used to identify specific coronaviral antibodies in blood serum and ascites fluid and was made using pork kidney cell culture, infected with coronavirus. Various dilutions of serum or ascites fluid were incubated with secondary antibodies (polyclonal mouse anti –cat IgG labeled with fluoresceine).

ELISA (enzyme-linked immunosorbent assay**)**

The indirect immunoenzymatic assay used to identify feline anticoronavirus antibodies in blood serum was EVL Feline Corona Virus antibody ELISA (F1005-AB02), according to the manufacturer‘s protocole. Optical density reading was performed using the ELISA Stat Fax 2200-2600 diagnostic line. As baseline: DO ≤ 30 negative (no antibody), 90-270 = positive (possibly canon coronavirus eliminator, requiring retest after 3 months), ≥810 = high titer (suggestive of PIF).

Results and discussion

For this study, we used various assays in order to highlight the feline coronavirus (FECV or FIPV) and the specific coronavirus antibodies. For FCoV detection, RT-PCR, DIF and IHC were used. The antibodies were identified using IFA and ELISA.

The samples collected from the 10 healthy cats (feces) were positive for EFCV using RT-PCR. DIF was not was not very suggestive, as fluorescence was not very specific (fig. 1). Also, FCoV antibodies detected with ELISA in blood serum showed positive titers, with optic density ranging between 18 and 245.

For the enteric FCoV infection, definitive diagnosis is necessary. In this case, antibody titers suggest prior exposure to the virus, but do not reflect fecal shedding or active infection. In the same time, the coronaviral particles identified in fecal specimens by RT-PCR have few implications for the health of an individual cat.

The samples collected from 10 domestic and wild felines (tiger) with clinical signs of FIP (wet form) were tested using all methods. FCoV was detected, using RT-PCR and DIF in all 10 samples of ascites fluid collected from alive felines (fig. 2). Also, DIF was positive in 4 intestine samples (fig. 3) and 2 kidney samples (fig. 4) harvested from corps.

The images obtained at DIF revealed a lot of cells (macrophages) with cell membrane fluorescence. Also, can be observed cells without the ring, but with fluorescence inside, perhaps internalized complexes (fig. 2). DIF on organ samples revealed fluorescence in enterocytes (fig. 3) or kidney macrophages (fig. 4).

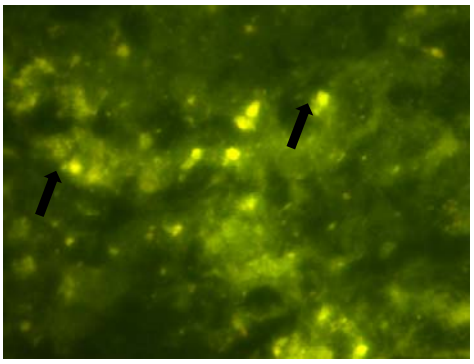

Fig. 1. Positive DIF in feces, x600

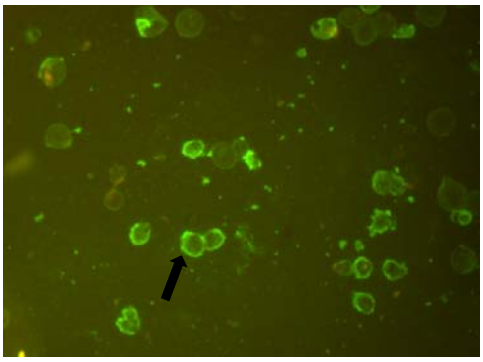

Fig. 2. Positive macrophages for FCoV DIF, x600

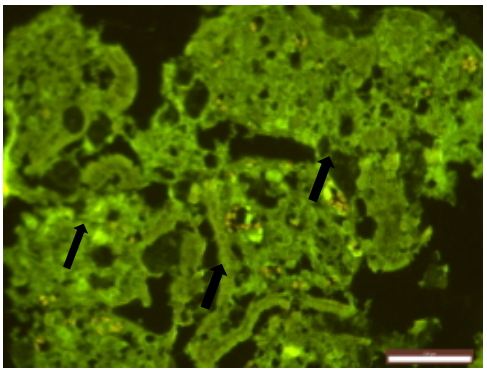

Fig. 3. Positive macrophages for FCoV in intestine, IHC, x600

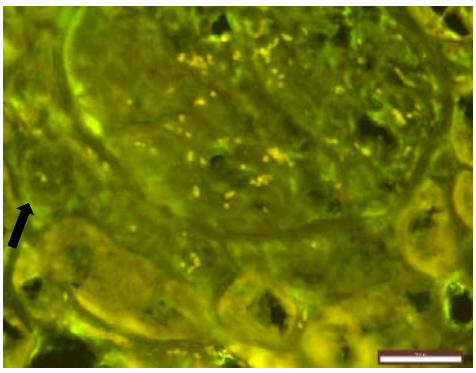

Fig. 4. Positive macrophages for FCoV in kidney, IHC, x600

Immunohistochemical testing of biopsy specimens and postmortem examination are the standard diagnostic methods. According some authors, DIF detected FCoV inside macrophages from effusion specimens with 100% specificity and has been recommended as an antemortem confirmatory test. Others calculated the sensitivity of DIF of 100% and the specificity about 71.4% (18) or 97% (3).

The IHC identified positive macrophages (brown) for FCoV in intestine (fig. 5) and in kidney samples (fig. 6).

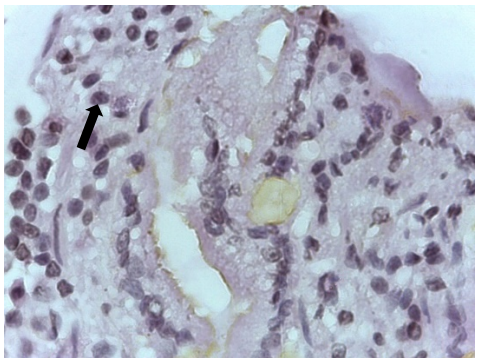

Fig. 5. Positive macrophages for FCoV in intestine, IHC, x600

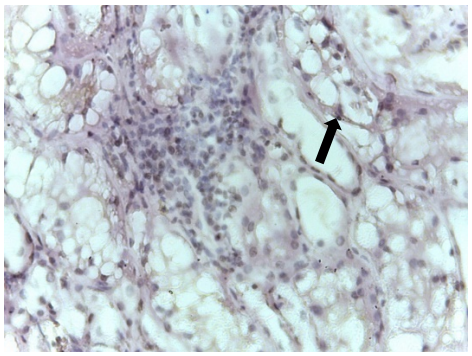

Fig. 6. Positive macrophages for FCoV in kidney, IHC, x600

Specific FCoV antibodies mesured by ELISA on serum / ascites fluid samples indicated values of optical density ranging from 926 to 3231, indicating a strong humoral immune response specific to PIF cats. Also, IFA indicated positive titers for FcoV antibodies with titers ranging from 1/125 to 1/16000.

The main purpose of this study to evaluate some diagnostic methods that can be used in routine diagnostic to confirm or infirm the diagnosis of coronavirus infection. These methods aimed at identifying the FCoV, specific FCoV antibodies or both of them in the clinical specimens. A clear distinction should be made between identifying coronaviral RNA in the feces and certifying the FIP diagnosis.

The 10 tested healthy cats (5 females and 5 males, different breeds and aged between 7 months and 3 years) came from a cattery in which the space was common, the food source and litter was shared. The identification of coronavirus in the feces in all felines was not a surprise, since the high density in the same space is a contributing factors. This control test confirmed this issue.

In the felines from the other category, the situation was different. First, nine of the cats (domestic) presented themselves with a general altered state, fever, some of them with anorexia, but all of them presenting a common symptom: ascites fluid in the abdominal cavity. Anamnesis, clinical, ultrasound, haematological and biochemical test results raised the suspicion of FIP evolution. The last case was the corpse of a 8 years old tiger, also suspected of FIP. Since this disease does not have a very specific symptomatology (especially the dry form), confirmation should be done with some specific tests

Confirmation of FCoV infection was done through the methods proposed for evaluation in this study. Although it is known that FIPV is a mutant of non-pathogenic and localized enterocyte pathogen (EFCV), differentiation of the two viruses can not be achieved (at least in routine testing). Moreover, it is demmonstrated that with the acquisition of the lethal potential, highly virulent

coronavirus acquires tropism for the macrophage monocyte system and loses enterocyte tropism (19, 21).

From this point of view, in living animals we need to use sensitive and specific methods that are capable of identifying one of the two coronaviruses in the pathological materials that we can harvest (blood, feces, ascitic fluid). Coronavirus identification in feces and lack of positivity in ascitic fluid is equivalent to refraining from FIP diagnosis, even if coronaviral antibodies are present at a 1/125 titre by IFA or DO≤90.

Various research articles provided variable results on the sensitivity and specificity of the immunofluorescence reaction directly performed on peritoneal fluids (3, 18). In our study, for example, all ascites fluid samples, harvested ante-mortem were positive, but unfortunately not all cats positive for FIP have reached necropsy for the post mortem exam. So, from this point of view, we could't certainly establish the sensitivity or specificity, but for sure we use this method as a first intension test.

To establish the PIF diagnosis with certainty, a multitude of tests, starting with clinical, ultrasound (nodular lesions within organs, organomegaly, effusions (pleural, peritoneal, pericardial, retroperitoneal), radiographic (body cavity effusions ± organo (megaly, pulmonary infiltrates) and continuing with paraclinical: haematological (nonregenerative anemia, neutrophilia ± left shift), biochemical (hyperglobulinemia with a low albumin: globulin ratio), elevated liver enzymes, azotemia, etc. Peritoneal and pleural fluid analysis is defining: are viscous, yellowish effusions with moderate cellularity (lymphocytes, macrophages, and nondegenerated neutrophils) (19, 20, 21).

Our samples of peritoneal effusion presented the classical yellow, viscous fluid gelling aspect observed when opening the corpse, due to high protein content. Also, fibrin deposits on nearly all organs in the abdominal cavity were other defining elements.

Serological tests for antibody identification are not very relevant for FIP diagnosis. Positive results are misleading because antibodies only show evidence of exposure to FCoV and are not specific for FIP or EFCV, but for FCoV. Moreover, many cats with no clinical signs, but with positive antibody titers never develop FIP. In other situations, cats, especially at the terminal stages of the disease, have a negative result. Only in cats with clinical signs of disease, the high antibody titer may have diagnostic value.

Identification of coronavirus RNA (FCoV) by RT-PCR may be performed for both FIPV and EFCV, but the test can not distinguish between the two entities. Also, the result may be negative for many cats. The positive results obtained in effusive samples are of certainty for FIP. In feces samples, however, it identifies only FCoV eliminators, which can be a source of infection for other animals.

If these tests can be performed on live animals, histopathology and immunohistochemistry are often performed on organ samples harvested from the cadavers to necropsies and can confirm or infirm the diagnosis. Therefore, we

recommend that the diagnosis of certainty be established after corroborating the anamnestic data, the clinical examination, possibly necropsy, and the results of the laboratory tests. Given the specificity of this disease, serological surveillance of predisposed cats (especially those in collectivities) and the elimination of favorable factors is essential. Positive serological results are not equivalent to the disease, but they signal that at any time the cat may be one of the 5% who develop FIP.

Conclusions

The evaluation of the methods used for the diagnosis of infections with FCoV allowed us to draw some conclusions, we say, important, first of all for the clinicians. Not every commercial test (lateral flow immunochromatography) is relevant to FIP, although the recommendations are for that purpose and are very easy to use. The vast majority of tests detect the presence or absence of the coronavirus antibodies without any reference to titre. Other tests are for FCoV detection in feces and are equally irrelevant for FIP. The most representative diagnosis for FIP is where we can identify FCoV in macrophages or monocytes. We recommend DIF test on puncture fluids (ascites, pleural). Positive macrophages for FCoV are equivalent to FIP diagnosis. In the case of a negative reaction, RT-PCR or real-time PCR can be used, but are more expensive and the owner may refuse. But these tests allow, after sequencing the amplicons, to perform a phylogenetic analysis to evaluate the strains of coronavirus circulating in our country. Antibodies titers can also be evaluated with commercial kitt or if the laboratory has facility for cell cultures and virus isolation, IFA can be performed. Immunohistochemistry is a very specific test, but is not a routine test.

Direct immunofluorescence is an alternative method to detect feline coronavirus in clinical samples, much faster and less expensive. Molecular biology offers instead the possibility to analyse the coronavirus strains circulating in Romania.

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LISTERIA SPECIES AND THEIR ENVIRONMENTALLY FREQUENCY OF ISOLATION IN A PORK PROCESSING ESTABLISHMENT: IMPLICATIONS FOR THE FOOD SAFETY

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Summary

Out from the seventeen species of the genus *Listeria*, only *L. monocytogenes* is recognized as causative agent of human listeriosis. The aim of the present study was to investigate the environmentally distribution and public health significance of *Listeria* species in a pork processing establishment in Romania. In this order, a total of 102 samples (65 from food contact and 37 from nonfood contact surfaces) were collected and processed according to the two-step enrichment ISO 11290/2000 A1/2005 standardized method, followed by species determination with the Vitek2 system. A detection rate of 77.5% (79/102) was registered for *Listeria* spp., with a distribution of 78.5% (51/65) on food contact and 75.7% (28/37) on non food contact surfaces, respectively. Three *Listeria* species were registered namely: *L. monocytogenes* (n=26; 32.9%), *L. welshimeri* (n=28; 35.4%) and *L. innocua* (n=25; 31.6%), respectively. No statistically significant differences ($p>0.05$) were recorded within the environmentally spreading of the recorded *Listeria* spp. The results of the current survey highlighted a large scale occurrence and moderate species diversity, including the human pathogen *L. monocytogenes*, in the monitored pork slaughtering and processing plant, with possible implications in the microbiological safety of the finished products, which can seriously threat the public health.

Keywords: *Listeria* spp., pork, environment, public health

The genus *Listeria*, included in the family Listeriaceae, comprises seventeen species namely *Listeria aquatica*, *L. booriae*, *L. cornellensis*, *L. fleischmannii, L. floridensis, L. grandensis, L. grayi, L. innocua, L. ivanovii, L. marthii, L. monocytogenes, L. newyorkensis, L. riparia, L. rocourtiae, L. seeligeri, L. weihenstephanensis* and *L. welshimeri.* Out of them, only *L. monocytogenes* can able to infect humans producing the disease namely listeriosis. *L. ivanovi* produce disease in animals. The most common transmission routes include the ingestion of contaminated foods and the pathogen vehiculation within the utero life from mother to fetus (2, 3, 7, 12).

Usually, results of several epidemiological surveys showed that *Listeria* species are widely distributed in the natural environments with possibility of detection in animal feed, soil and vegetation (reviewed by 15). Also, the pathogen can survive under various conditions in non-host habitats, which make difficult to its

control. Regarding the food contamination, it can occur at any level of the food chain (farm-processing-retail) or even at the home of the consumer (11). The conventional food treatments (e.g. pasteurization, cooking) can easily inactivate *Listeria*, but it is capable to survival in chilling, dehydration and even freezing conditions. Several investigations pointed out that this pathogen remains an important problem in the case of ready-to-eat foods safety, considering that several cross-contamination ways can occur within the food processing environment before packaging, even in the case of the previously thermal processed foods (2, 13).

Listeria is a very adaptable pathogen and capable to growth under various foods processing conditions (e.g. temperature from 1°C to 45°C; ph from 4.3 to 9.5; water activity > 0.90; high NaCl concentrations, up to 10%) with ability to colonize and multiply on food processing equipments for a long time (2, 3, 4). Taking these considerations, the knowledge of the environmental spreading of the pathogenic *Listeria* spp. in each production unit can significantly help food safety managers to improve their HACCP plan and sanitation programs in order to minimize the health risk to the consumer.

The aim of the present study was to investigate the environmentally distribution and public health significance of *Listeria* species in a pork processing plant from Romania.

Materials and methods

In a pork processing establishment situated in western Romania, during a period of 11 months, a total of 102 samples (65 from food contact and 37 from nonfood contact surfaces), consist on five grouped sterile cotton swabs rubbed over a surface of approximately 100 cm^2, were collected during the employers operations. The labeled sterile bags containing the collected swabs were shipped on the collection day to the microbiology laboratory for analysis under refrigeration conditions.

In order to detect *Listeria* spp. the collected samples were processed according to the two-step enrichment SR EN ISO 11290/2000 A1/2005 standardized method (8). The working methodology included the following successively and complementary main steps: (*i*) pre-enrichment and incubation at 30 ^{o}C for 24 h in half-Fraser broth; (*ii*) secondary enrichment and incubation at 37 ^{o}C for 48 h in full-Fraser broth; (*iii*) streaking of the pre-enriched and enriched broth cultures onto ALOA selective medium, followed by incubation at 37 ^{o}C for 24 and 48 h; sub-culturing of up to five presumptive *Listeria* colonies from the each enrichment step on tryptone soya agar containing yeast extract (8). Subsequently, the growed *Listeria* colonies were Gram stained and examined with motility, catalase and oxidase tests, followed by the detailed biochemical testing of the Gram, catalase and oxidase positive isolates with the Vitek2 automated system (bioMérieux, Marcy-l'Etoile, France). Also, within the Vitek2 analysis the *Listeria* species were determined. In addition, in case of the identified *L. monocytogenes*

isolates their identity was molecularly confirmed, targeting the species specific *hly*A gene and using the methodology described by Lawrence and Gilmour (1994) (6).

The nonparametric Pearson's Chi-square test was use for statistical analysis of the obtained data and a $p<0.05$ value was considered significant.

Results and discussions

The study results are summary presented in Table 1.

Overall, from the total of 102 examined samples, 26 (25.5%) were positive for *L. monocytogenes*, 28 (27.5%) for *L. welshimeri*, and 25 (24.5%) for *L. innocua*. No *Listeria* spp. was identified in 23 (22.5%) samples. A relatively uniform distribution of the registered species on food contact and nonfood contact surfaces was observed (Table 1). Within the food contact surfaces the highest detection rate (100%) for *Listeria* spp. was registered in the case of processing equipments and storage containers. The human pathogen *L. monocytogenes* was most frequently isolated from conveyor belts and processing equipments (Table 1). In the case of nonfood contact surfaces the slaughterhouse/processing plant drains were the most *Listeria* contaminated, with dominance of *L. monocytogenes*.

Table 1

Environmentally distribution of the identified *Listeria* spp. in the screened pork processing establishment

Sample origin (*n*)	**Isolation source (no. of samplings)**	**No. of positive *Listeria* spp. (%)**			
		L. monocytogenes	***L. welshimeri***	***L. innocua***	**Absent**
Food contact (65)	conveyor belts (15)	5 (33.3)	5 (33.3)	4 (26.6)	1 (6.7)
	cutting surfaces (8)	2 (25.0)	3 (37.5)	1 (12.5)	2 (25.0)
	packing surfaces (6)	1 (16.7)	1 (16.7)	2 (33.3)	2 (33.3)
	personnel equipment (7)	1 (14.3)	1 (14.3)	1 (14.3)	4 (57.1)
	processing equipment (11)	3 (27.3)	5 (45.4)	3 (27.3)	0
	slaughter equipment (12)	3 (25.0)	0	4 (33.3)	5 (41.7)
	storage containers (6)	0	5 (83.3)	1 (16.7)	0
Nonfood contact surfaces (37)	employee workflow areas (8)	2 (25.0)	2 (25.0)	1 (12.5)	3 (37.5)
	slaughterhouse / processing plant drains (20)	8 (40.0)	2 (10.0)	7 (35.0)	3 (15.0)
	cooling chamber walls (9)	1 (11.1)	4 (44.4)	1 (11.1)	3 (33.3)
Total	**102**	**26 (25.5)**	**28 (27.5)**	**25 (24.5)**	**23 (22.5)**

No statistically significant associations were recorded between the registered *Listeria* spp. and their distribution on food contact and non food contact surfaces.

To complete a previous survey conducted by Sala et al. (2016) (10), the present paper adds data on the knowledge of the *Listeria* species spreading in the food processing environment. Also, according to the authors knowledge this is the first report in Romania confirming the occurrence of *L. innocua* and *L. welshimeri* in meat processing environment.

The screening and identification of non-pathogenic *Listeria* spp. in the food production units play an important role, because their presence can act as markers for the possible occurrence of the human pathogen *L. monocytogenes*. Moreover, Rocourt and Buchrisier (2007) (7) pointed out that the species *L. monocytogenes* and *L. innocua* frequently share the same ecological niche. According to Vasu et al. (2014) (14) the recovery rate of *L. innocua* could be considered as useful indicator for the possible occurrence of *L. monocytogenes*, with a shorter generation time.

The highest frequency of isolation of *Listeria* spp., with dominance of *L. monocytogenes*, registered at the level of plant drains; suggest that the routine pre- and post-operational sanitization procedures are not enough to eliminate the pathogen from the environment. Also, from this level a potential cross-contamination to food contact surfaces can occur. The relatively high isolation rate of *L. monocytogenes* from several food contact surfaces (e.g. two of the focal points of the plant - conveyor belts 33.3%, processing equipment 27.3%) highlights their role as contamination source of the finished products. This fact can be mainly realized through persisting of the pathogen in the processing environment in different biofilm structures. Several authors concluded that the food contact surfaces of the processing plants represent more important *L. monocytogenes* sources than raw materials, even if the raw materials are reported as initial sources of the pathogen (1, 2, 5, 9).

In agreement with the results obtained in the present study, the large scale distribution of *L. innocua* and *L. welshimeri* and other *Listeria* spp., beside *L. monocytogenes*, has been confirmed by Williams (2010) (15), during a one year longitudinal study conducted in six small and very small meat processing units. The prevalence of the identified species ranged from 1.5 % to 18.3% across the investigated units. In another study conducted by Autio et al. (2000) (1) in Finland, the frequency of isolation of other *Listeria* spp. than *L. monocytogenes* from different pork processing environmental sources was 40% in environment drains, 10% environment drains and doors and 0% on knives.

The obtained results highlighted that the registered in house – *Listeria* flora in the screened unit can constitute a potential health risk for the consumer. Also, the recorded *Listeria* contamination rate in the working environment and food processing equipments, as possible source of contamination for finished products, point out the requirement of special attention during the sanitation and cleaning procedures.

In addition, in order to implement effective hygiene procedures on the basis of HACCP principles, further studies are needed to complete and/or reconsider the analysis of critical control points of the unit.

Conclusions

The results of the current survey highlighted a large scale occurrence and moderate *Listeria* species diversity in the monitored pork processing establishment.

The identification of the human pathogen *L. monocytogenes* pointed out possible implications in the microbiological safety of the finished products.

The survey offer useful information for food safety managers of the unit in order to improve their implemented HACCP programs.

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LONG TERM STUDY OF THE SPATIAL EXPANSION OF CANINE BABESIOSIS IN TIMIȘ COUNTY

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Summary

Knowledge of the continuous expansion and territorial distribution of the tick borne diseases and their causative agents is important for the veterinary practitioners and for animal owners. Canine babesiosis was identified in many European countries including Romania. The spreading of the disease in some regions and lack of clinical cases in others, suggests a regional endemicity influenced by the presence of infected tick populations. This study aimed to map the spatial distribution of *Babesia* spp. cases in Timiș County and Timișoara metropolitan area using the geographical information system (GIS). Data about clinical cases, including genetic characterization of causative agents at species level, were collected from the Clinic of Parasitology and Parasitic Diseases of the Faculty of Veterinary Medicine Timisoara between 2010 and 2017. The geographical location of the cases was recorded using a GPS device and were analyzed through a GIS software. Out of the 51 recorded cases, 45 (88.2%) were infected with *B. canis*, 5 (9.8%) with *B. gibsoni* and 1 (1.9%) with *B. vogeli*. The majority of the cases were located in metropolitan areas of Timișoara (*n*=43). The resulted graphics describe the exact location of the *Babesia* positive samples, with a high number of cases near parks and in urban areas. The regional endemicity of the disease can offer useful information for veterinarians and dog owners in their attempt to prevent and control this disease. Owners from the endemic regions are advised to avoid tick-contaminated areas and to apply tick preventive treatment on their dogs.

Keywords: *Babesia*, spatial distribution, dogs

Canine babesiosis is a wide spread tick-borne disease caused by small and large *Babesia* species. The geographical distribution of the species, transmission of pathogens, clinical signs and specific treatment may vary between the species involved. There are three large *Babesia* species namely, *Babesia canis*, *Babesia vogeli* and *Babesia rossi*. Presently, only the first two have been recorded in our country (4, 7, 8, 11). Small species include *Babesia gibsoni*, *Babesia comrade*, *Babesia vulpes* previously noted as *Babesia* "Spanish dog

isolate", *Babesia microti*-like and *Babesia (Theileria) annae* (1, 3, 13, 15). *Babesia gibsoni* is the most spread out of the small species identified worldwide (13). The identification of these pathogens can be carried out based on blood smear examination, useful in the case of large *Babesia* species and in small laboratories or veterinary clinics and by more specific and sensitive molecular methods, used in specific laboratories (10, 13).

Geographical expansion is important in canine babesiosis since the disease has a regional evolution depending on the spreading of the infected tick host. Veterinary practitioners are the first to notice the emergence of the cases and to adopt the preventive measures in case of new outbreaks. Location of the animal can influence his health and the evaluation of regional risk is imperious in establishing a plan for disease prevention and control. As in case of babesiosis, tick hosts (*Dermacentor reticulatus* and *Rhipicephalus sanguineus)* are widely spread in Timiș County in dogs (9), thus the evolution of the disease in this area can be anticipated.

This study aimed to map the spatial distribution of clinical cases of canine babesiosis in Timiș County and Timișoara metropolitan area using geographical information system (GIS).

Materials and methods

Registered cases of canine babesiosis from the Clinic of Parasitology and Parasitic Diseases of the Faculty of Veterinary Medicine Timișoara between 2010 and 2017 were collected and analyzed regarding the geospatial location of the infected animal. The cases contain data about genetical characterization of the pathogen to establish *Babesia* species involved in the infection. All 51 cases were molecularly characterized using PCR and PCR-RFLP protocols previously described by Imre et al 2017 and Solano-Gallego et. al. 2008 (10, 14). The majority of cases were located in metropolitan areas of Timișoara (*n*=43) and in neighboring localities (*n*=8).

The geographic location of the cases was acquired with a GPS device, and a spatial analysis (6) of these cases was carried out via GIS dedicated software: ArcGIS and ArcGIS Online (5). The spatial distribution of cases that were recorded in the field can be seen in the figure below.

Results and discussions

The location of the positive samples is presented in Fig. 1 and Fig. 2.

Out of the 51 recorded cases, 45 (88.2%) were infected with *B. canis*, 5 (9.8%) were infected with *B. gibsoni* and 1 (1.9%) was infected with *B. vogeli*. The majority of the cases were located in metropolitan areas of Timișoara (*n*=43). The graphics describe the exact location of the *Babesia* positive samples, with a high number of cases near parks and in urban areas.

The location of positive clinical cases from the metropolitan area of Timișoara is presented on the first map. The red marked area represents the positive cases of *B. canis* and the dominance (38 cases) of this large species can be observed in the evaluated region. A smaller number of cases of *B. gibsoni* (5 cases, blue dots) were diagnosed in this area and *B. vogeli* was not identified. We can observe a wide distribution of cases of *B. canis* in the studied area. Although the disease is distributed in all regions of Timisoara city, they are more frequent in the south -eastern part of the city near one large park. The presence of positive cases near parks is expected as the main species of ticks that transmits this pathogenous agent is *Dermacentor reticulatus*. The habitat of this tick is in most of cases parks from metropolitan areas.

Fig 1. The GIS location of positive clinical cases from the metropolitan area of Timișoara

The GIS map from figure 2 presents the cases registered from non metropolitan areas of Timiș County. As it can be observed, the cases are not that frequent and there is a dominance of *B. canis* cases (red dots). Also, one case of *B. vogeli* (Green dot) was registered in the western part of the county.

In the urban areas, the number of cases can be higher than in rural areas as the access to public parks is easier, the number of animals in the park is larger and the contact between animals and between animals and ticks is more frequent.

In the localities of Timiș County, a dog owner usually has a backyard and the dog does not have access outside the yard. Limited access to public areas also limits the contact with infected ticks. In metropolitan areas, dogs are frequently walked in public parks and owners frequently travel with pets to different locations. Thus, the risk for contacting ticks and babesiosis is higher.

A higher number of clinical cases of babesiosis in the last years, and also a higher number of ticks can be attributed to climate change. Weather conditions influence the number of ticks and the activity periods of ticks. The decrease of winter days with a milder and shorter winter period affects the lifetime, survival and questing activity of ticks and transmission of the disease in Europe (2).

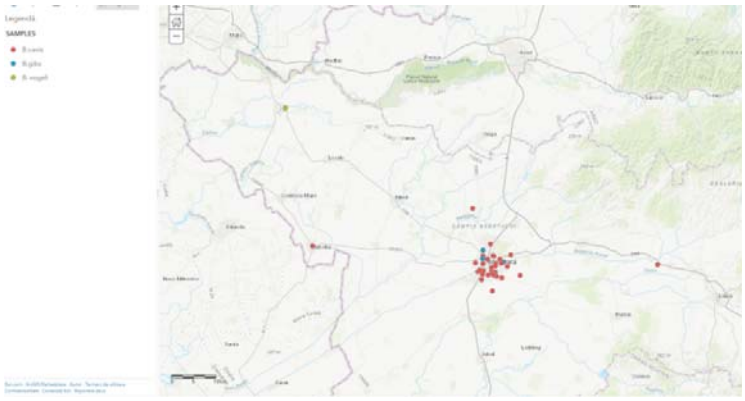

Fig 2. The GIS location of positive clinical cases from Timis County

Local incidence of babesiosis in Europe was documented in different regions and countries. *B. canis* is the main pathogen, with different local variations in France, Spain, Hungary, Italy, United Kingdom, Germany, Switzerland and Austria emphasizing the importance of tick control in dogs in endemic regions (12).

The regional endemicity of the disease can offer useful information for veterinarians and dog owners in their attempt to prevent and control this disease. Owners from the endemic regions are advised to avoid tick-contaminated areas and to apply tick preventive treatment on their dogs.

Conclusions

Babesiosis is spread in Timis County with higher frequency in urban areas.

The highest number of cases registered in the south – eastern part of Timisoara metropolitan area suggest the local spreading of the disease

Walking of the dogs in public parks with high abundance of ticks is a risk factor for babesiosis

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CARYOSPORA (AVISPORA) SPECIES INFECTING FALCONS IN THE UNITED ARAB EMIRATES

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Summary

Caryosporosis in captive bred falcons is one of the most important causes of morbidity. High infection decreases the ability of falcons to perform well in training, hunting and competitions. Extreme weight loss and even death may occur in severe cases. This study identified the species that infect falcons in the United Arab Emirates. They were *C. kutzeri, C. neofalconis, C. megafalconis, C. falconis, C. cherrugi,* and *C. boeri.*
Keywords: *Caryospora(Avispora), falcons*

There are nine *Caryospora* species described in specialty literature, namely *C. boeri, C. biarmicusis, C. cherrugi, C. henryae, C. kutzeri, C. neofalconis, C. megafalconis, C. falconis* and *C. peneiroiroi* (1,2,3). The life cycle takes seven to 13 days to complete (4,5) and if the birds receive treatment during this period, they can develop an immune response (6).

The presence of *Caryospora spp*. has been demonstrated in free-living birds of prey (6), although the highest prevalence occurs in captive bred falcons (8). These studies demonstrated that the prevalence of caryosporiosis varies from 12 to 86% (9).

The clinical signs of *Caryospora spp*. infection in falcons consist of abdominal cramps, lethargy, fluffed up appearance, weight loss, loss of appetite, and death in severe cases (5,6).

Caryosporiosis is a challenging infection in what regards control, due to the lack of immunity in young falcons and to the persistence of oocysts in the environment (9).

The objective of this study was to identify the species of *Caryospora* in falcons present within the UAE based on morphological characteristics.

Materials and methods

The study was conducted in the period September 2015- February 2016. A number of 375 Falcon fecal samples were collected and microscopically evaluated. The samples tested positive for the presence of *Caryospora spp.* were studied.

The Falcons included in the study were bred in captive breeding centers from within the UAE and abroad. The falcon species taken into study were: the gyr falcon (*Falco rusticolus*), peregrine falcon (*Falco peregrinus*), gyr x peregrine hybrid (*Falco rusticolus x Falco peregrinus*), saker falcon (*Falco cherrug*), gyr x saker hybrid falcons (*Falco rusticolus* x *Falco cherrug*), Eurasian kestrel, red-napped shaheen (*Falco pelegrinoides babylonicus*), black shaheen (*Falco peregrinus peregrinator*), lanner falcon (*Falco biarmicus*), American kestrel (*Falco sparverius*), and Eurasian hobby (*Falco subbuteo*).

Samples were processed in the Clinical Diagnostic Laboratory of the Wildlife Division, Wrsan. The methodology included microscopic examination, sporulation, oocysts measurements, and photography. The species determination was performed morphometrically (3).

Fresh fecal samples were collected in 60 mL plastic tubes, containing potassium dichromate solution ($K_2Cr_2O_7$) (10). They were allowed to sporulate. Sporulation ended within 72 – 94 h at 22±2°C. Samples were examined using Sheather's sucrose solution flotation method. The oocysts were collected under a coverslip for 20 minutes in 5 mL plastic tubes. The coverslip was lifted and placed onto a slide for microscopic examination (Olympus BX41, with a viewing camera Olympus DE71). The oocysts were studied using 10x magnification and measured at 40x and 100x magnification using a calibrated ocular micrometre (Nikon Japan eyepiece adapter CFIUW 10x/25). The morphologic characteristics of the oocyst and structures were determined in micrometres (µm) (2,5).

Results and discussion

The *Caryospora spp.* identified in this study were *C. kutzeri, C. neofalconis, C. megafalconis*, *C. falconis*, *C. cherrugi,* and *C. boeri.* The measurements of the species obtained are presented in Table 1.

The falcon species infected were: gyr falcon (*Falco rusticolus*), peregrine falcon (*Falco peregrinus*), gyr x peregrine hybrid (*Falco rusticolus x Falco peregrinus*), saker falcon (*Falco cherrug*), gyr x saker hybrid falcons (*Falco rusticolus* x *Falco cherrug*), Eurasian kestrel, red-napped shaheen (*Falco pelegrinoides babylonicus*)

Infection with two to three different *Caryospora* spp. was identified in the same falcon specimen. *C. kutzeri* and *C. neofalconis* were identified in peregrine falcons. In other specimens three *Caryospora* spp. were identified in the same

peregrine falcon, namely *C. kutzeri, C. neofalconis and C. falconis*. In gyr falcons, the presence of *C. kutzeri* and *C. neofalconis* was observed in the same specimen.

Table 1

***Caryospora* spp. and morphological characteristics reported in this study**

***Caryospora* spp.**	**Oocyst shape**	**Oocysts size (µm)**	**Sporocyst Shape**	**Sporocyst size (µm)**	**Sporozoite size (µm)**	**Host**
C. kutzeri	Subspherical	39.1 x 33.1 (33-46 x 28-40)	Ovoid	22.5 x 21.3 (18-26 x 16-26)	4.6 (2-7 x 2-7)	*F. rusticolus F. peregrinus F. cherrug, F. tinnunculus*
C. neofalconis	Subspherical	25.6 x 22.5 (20-30 x 19-28)	Ovoid	17.2 x 15.2 (13-19 x 12-19)	9.9 x 2.4 (8-12 x 2-4)	*F. rusticolus F. peregrinus F peregrinus pelegrinator F. cherrug, F. sparverius*
C. megafalconis	Subspherical	42.2 x 35.9 (39-49 x 30-40)	Spherical	23.6 x 22.7 (21-27 x 20-26)	16.5 x 4.2 (11-20 x 3-6)	*F. rusticolus, F. peregrinus F. cherrug*
C. falconis	Spherical	32.4 x 29.8 (29-36 x 23-35)	Spherical	21.7 x 20.6 (15-25 x 14-25)	13.2x4.6 (10-17 x 2-4)	*F. peregrinus*
C. cherrugi	Ovoid	33.7 x2 8 (29-35 x 23-32)	Ovoid	22.6 x 18.9 (20-25 x 15-22)	15 x4 (12-20 x 3-5)	*F. cherrug*
C. boeri	Subspherical	38.5 x 31 (39 x 30-32)	Ovoid	24.5 x 20.5 (24-25 x 20-21)	19 x 3.5 (18-20 x 3-4)	*F. tinnunculus*

Conclusions

Six out of nine *Caryospora* species mentioned in literature were identified in this study. This was facilitated by direct transmission of *Caryospora* spp.' which takes place when falcons from all over the world are brought together Overcrowding, poor hygiene, handling and feeding on the same glove usually occur in falcon sale centers, making infection more probable.

Acknowledgements

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STUDY ON THE IDENTIFICATION OF ENDOPARASITOSIS IN REPTILES KEPT IN CAPTIVITY FROM THE PET SHOP IULIUS MALL TIMISOARA AND THEIR ROLE IN HUMAN CONTAMINATION

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Summary

Reptiles can be hosts for different parasite species and can be able to determine diseases in domestic animals or even humans, their role as a natural reservoir is well known. The aim of this study is to identify possible species of parasites in reptiles kept in captivity that show clinical signs or not. The study was carried out on a group of 120 turtles, 10 iguanas, 9 snakes, 6 gecko, 4 agama beards from the Pet Shop Iulius Mall Timisoara. Coproscopic examination performed at 149 reptiles identified *Protozoa* oocysts and nematode eggs (*Kalicephalus spp., Oxyuris spp*.). The parasite species identified in captive reptiles with clinical signs were: *Isospora spp*. and *Oxyuris spp.* The parasite species found in captive reptiles that did not show any clinical signs were: *Isospora spp*. and *Kalicephalus spp.* This study on captive reptiles is important from parasitological point of view: the parasites can cause serious illness, even the death of these pets, and some of them may represent a risk of human contamination.

Keywords: captive reptiles, parasites, human contamination

Nowdays, the study of parasitic fauna in reptiles remains a current and attractive field. Researchers are concerned about new information regarding the biology and pathology of wild reptiles, but especially those kept in captivity (1, 3, 4, 5).

Reptiles can be hosts for different parasite species and can be able to determine diseases in domestic animals or even humans, their role as a natural reservoir is well known (16).

In the case of reptiles kept in captivity, a parasitological control program can be established, the parasitoses diagnosed being thus easily controlled by the veterinarian. But, the epidemiology of parasitic diseases in these reptiles is complicated when reptiles caught in the wilderness are sold, these reptiles being parasitic with species not normally found in reptiles kept in captivity.

In this situation, the control of these parasites will be difficult to achieve (17, 18).

Reptiles as pets are increasingly purchased in pet shops. These include snakes, iguanas, gecko, chameleons and turtles. Deworming reptiles is as important medical act just like other pets (dog, cat).

The aim of this study is to identify possible species of parasites in reptiles kept in captivity that show clinical signs or not.

Materials and methods

The study was carried out on a group of 120 turtles, 10 iguanas, 9 snakes, 6 gecko, 4 agama beards from the Pet Shop Iulius Mall Timisoara.

Of the total number of reptiles (149), a total of 34 showed clinical signs. These include 8 iguanas, 4 gecko, 7 snakes, 4 beards agama and 11 turtles (fig. 1, 2). Reptiles showed digestive signs, diarrhea, apathy, and progressive weight loss. These represent the first study group.

Lot II was made up of 115 reptiles with no clinical signs.

Coproscopic examinations (Willis method) have been performed to identify possible parasites and thus prevent clinical signs from being triggered.

The reptiles were between 10 days and 2 years old.

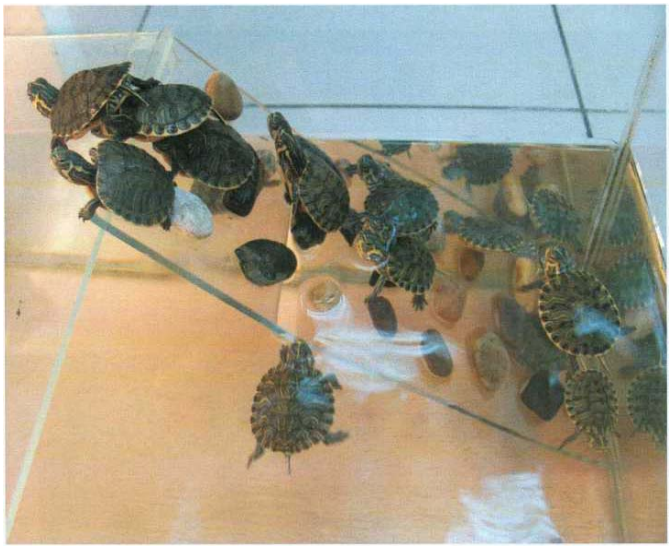

Fig. 1. Turtles from Iulius Mall Timisoara

Fig. 2. Iguana – Mall Timisoara

The faeces harvested from 149 reptiles was examined trough the Willis method.

Protozoa oocysts and nematode eggs (*Kalicephalus spp., Oxyuris spp*.) have been identified (fig. 3, 4).

The results of the coproscopic examinations for both groups (with clinical signs and no clinical signs) indicated a high infestation with *Isospora spp*. (Table 1, 2).

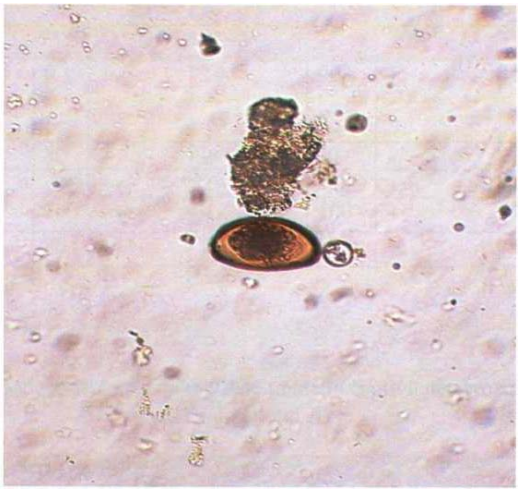

Fig. 3. Nematod egg and protozoa oocyst

Fig. 4. Nematod eggs

Table 1

Results of coproscopic exams in captive reptiles with clinical signs (group I)

Group I	Total	Positives	%	*Isospora spp.*	Positives	%	*Oxyuris spp.*	Positives	%
Turtles	120	<11	9.1	+++	10	90	+	10	9,1
Gecko	6	4	80	+	2	50	-	-	-
Snackes	9	7	77	++	2	28	+	1	50
Agama beards	4	4	100	+++	4	100	+	1	25
Iguanas	10	8	80	+++	2	80	+	1	50

Table 2

Results of coproscopic exams in captive reptiles without clinical signs (group II)

Group II	Total	Positives	%	*Isospora spp.*	Positives	%	*Kalicephalus spp.*	Positives	%
Turtles	120	109	89	+++	80	73	+	10	9,1
Gecko	6	2	33	+	2	100	-	-	-
Snakes	9	2	22	++	2	100	+	1	50
Iguanas	10	2	20	+++	2	80	+	1	50

The results of group I releved the parasitism with *Isospora spp*. as folows:
• to turtles - high infestation (+++)
• to gecko - poor infestation (+)
• to snakes - medium infestation (++)
• to beard agama – high infestation (+++)
• to iguanas – high infestation (+++).

Group I was parasited with *Oxyuris spp.* as follows:
• to turtles - poor infestation (+)
• to gecko - absent (0)
• to snakes - poor infestation (+)
• to beard agama – poor infestation (+)
• to iguanas – poor infestation (+).

Coproscopic examination results in reptiles of group II infected with *Isospora spp*were the following:
• to turtles - high infestation (+++)
• to iguanas - high infestation (+++)
• to gecko – poor infestation (+)
• to snakes – medium infestation (++)

In the second group, the infestation with *Kalicephalus spp*. presented the following levels:
• to turtles - poor infestation (+)
• to iguana – poor infestation (+)
• to gecko - absent
• to snakes- poor infestation (+).

In group I*, Isospora spp.*infestation had the highest prevalence, being identified in 11 turtles, 8 iguans, 7 snakes, 4 gecko and 4 beard agama (fig. 5).

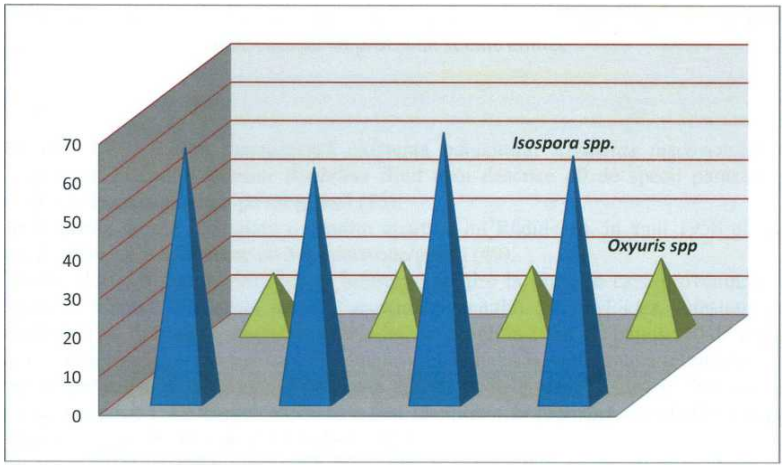

Fig. 5. The prevalence of *Isospora spp* and *Oxyuris spp* infestation – group 1

A lower incidence was found in *Kalicephalus spp*. infestation, being recorded in all species of reptiles, except for gecko from group II (fig. 6).

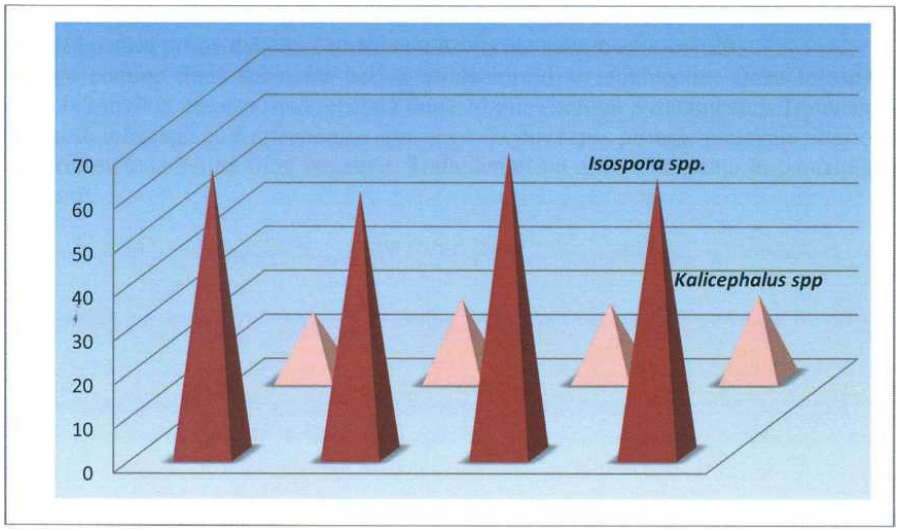

Fig. 6. The prevalence of *Kalicephalus spp.* infestation – group 2

In 1899, Raillet referred to the existence of the *Rhabdias fuscovenosa* in the snakes' lungs, later in the body of amphibians and reptiles spread all over the globe (2).

In Romania, the first report of gender *Rhabdias* belongs to Radulescu, in 1958.

The nematode *Eustrongylides exisus* with zoonotic potential was identified first time in the world, in snakes, by Mihalca. The authors identified in the small intestine of the house snakes from St. George location parasites belong to the gender *Strongyloides* (15).

Studies on reptiles parasites were conducted in Mauritius, Ecuador, Guyana, Paraguay, Peru, Cosata Rica, Thailand, Brazil and Costa Rica (9, 10, 11, 12, 13, 14, 19, 20).

The first treatment of infestation with *Kalicephalus spp.* and *Oxyuris spp.* has been successfully performed in Romania by Groza Ama used Advocate spot-on (6).

Conclusions

Coproscopic examination performed at 149 reptiles identified *Protozoa* oocysts and nematode eggs (*Kalicephalus spp., Oxyuris spp*.).

The parasite species identified in captive reptiles with clinical signs were: *Isospora spp*. and *Oxyuris spp.*

The parasite species found in captive reptiles that did not show any clinical signs were: *Isospora spp*. and *Kalicephalus spp.*

In group I (reptiles kept in captivity showing clinical signs), *Isospora spp*. was the most prevalent parasite, and *Oxyuris spp*. had the lowest prevalence.

In group II (reptiles kept in captivity without clinical signs), *Isospora spp*. was also the highest prevalent parasite and *Kalicephalus spp*. had the lowest prevalence.

This study on captive reptiles is important from parasitological point of view: the parasites can cause serious illness, even the death of these pets, and some of them may represent a risk of human contamination.

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HOW USEFUL ARE SIGNALMENTS TO DIAGNOSE OTITIS EXTERNA IN DOGS AND CATS

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Summary

Otitis externa, a frequent condition of the ear canal, prevalent in dogs and cats, is characterized by erythema and increased epithelial desquamations, by abundant ceruminous discharge and by variations of the pain and pruritus. The main clinical signs, reported by owners were head shaking and pruritus and clinically, the most relevant sign were the dark-coloured secretions found in 100% of dogs and 88.88% of cats**.** In dogs, otitis externa of bacterial and mycotic origin are more frequently noticed, while in cats the parasitic and bacterial ones are more prevalent
Keywords: otitis, dog, cat, secretions

Otitis externa is the most frequent illness of the ear canal in dogs and cats, being an acute or chronic inflammation of the external acoustic meatus epithelium (1, 4, 7). It can develop anywhere, beginning from the tympanic membrane to the pinna. This condition is characterized by the erythema and increased epithelial desquamations, by abundant ceruminous discharge and by variations of the pain and pruritus.

This paper tries to present the most common otitis externa cases in dogs and cats alongside with the identification of certain clinical signs which can lead to a doubtless diagnosis in a very short time.

Materials and methods

Twenty cases, 11 dogs and nine cats, were taken into consideration from a private veterinary clinic from Timisoara, aged from 6 months to 11 years.

Besides the history and the clinical examination, some specific dermatological exams, such as otoscopy or cytology, were performed.

Results and discussion

Age predisposition was noticed, namely the most affected was the over 3 year-old category (54.54% in dogs and 66.67% in cats), but no sex predisposition was seen.

Concerning the history, six valid findings for otitis externa have been considered in dogs (Fig. 1). Thus, the most frequent findings were head shaking (72.72%) and pruritus (63.63%), while complications such as furunculosis had the lowest prevalence (9.09%).

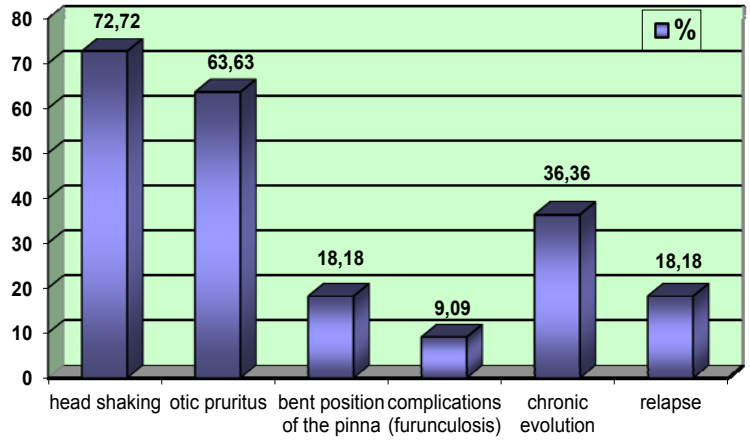

Fig 1. Contribution of the main historical data to diagnose otitis externa in dogs

The analysis of clinical data was based on the presence of brown-blackish secretions, pain, erythema, proliferation, external ear canal inflammation, external ear canal induration and narrowing, external ear canal hairiness, pseudo-clipping and pus.

All these changes had a relatively wide distribution. Dark secretions were seen in all investigated dogs (100%) being the most representative sign, followed by erythema and external acoustic meatus induration and narrowing, both with a frequency of 27.27% (Fig. 2). Considering these remarks, it can be asserted that all these highly prevalent signs represent the main clinical benchmarks in otitis externa.

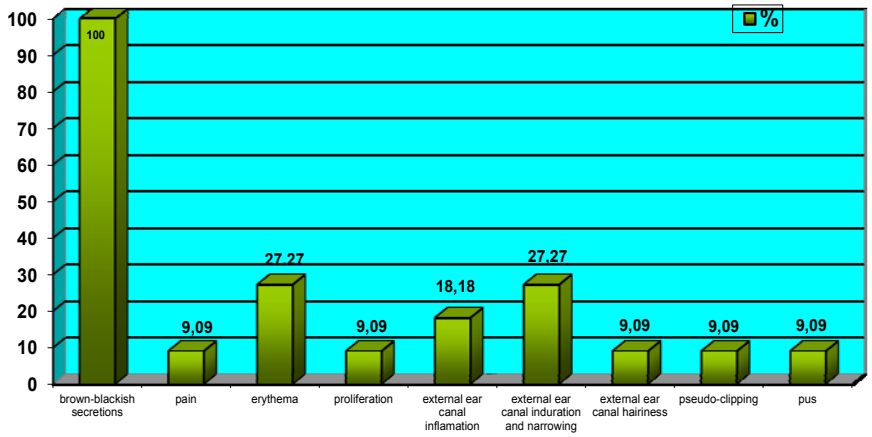

Fig. 2. Contribution of the main clinical signs to diagnose the otitis externa in dogs

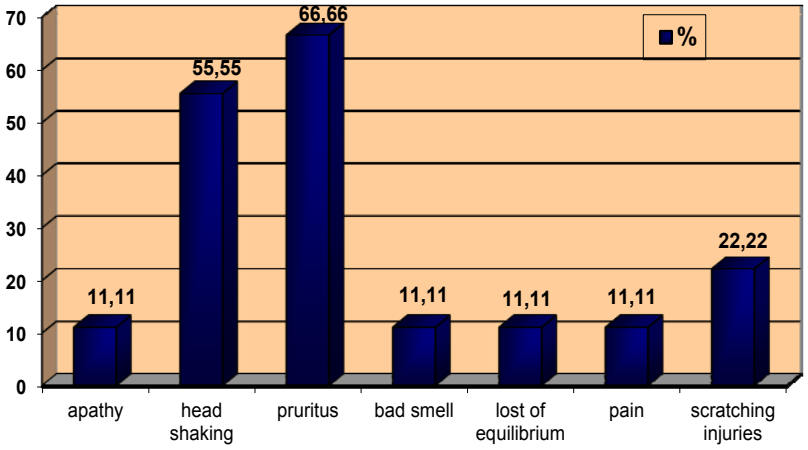

Fig. 3. Contribution of the main anamnestic data to diagnose otitis externa in cats

Only three diagnoses were established for these cases: parasitic otitis (18.18%), and both bacterial and mycotic otitis (72.72%), respectively.

In cats, seven valid findings for otitis externa have been considered (Fig. 3). Unlike dogs, pruritus was the most prevalent sign (66.67%), followed by head

shaking (55.55%) and scratch injuries (22.22%). All the other findings had a prevalence of 11.11% each.

The considered clinical data were less numerous than those observed in dogs (six out of nine). The brown-blackish secretions were more prevalent (88.88%), while the other signs had a frequency of 11.11% in all investigated individuals (Figure 4).

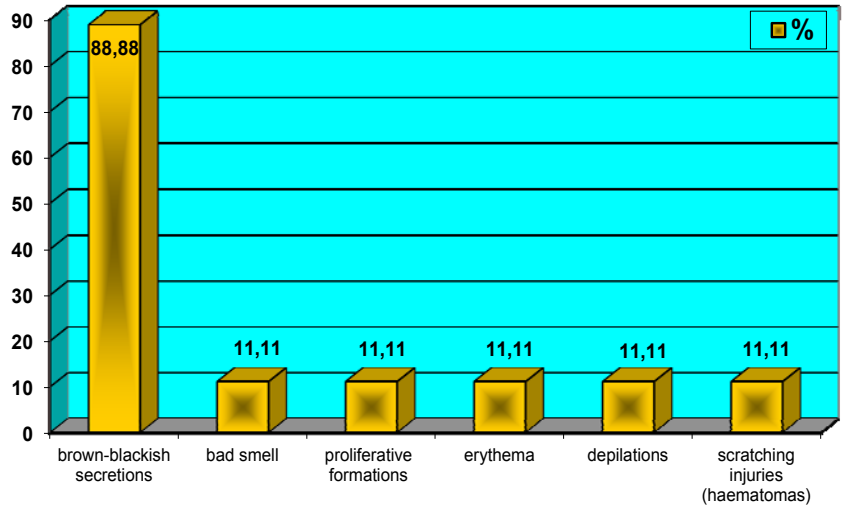

Fig. 4. Contribution of the main clinical signs to diagnose otitis externa in cats

Compared to dogs, the parasitic otitis was more frequent in cats (44.44%). However, its prevalence was exceeded by the bacterial otitis (55.55%), while mycotic otitis had only 33.33%, half the prevalence observed in dogs.

Even if most authors indicate an increased prevalence of otitis externa in cats, between 50% and 80% of the total otitis externa cases (2, 3, 6), the data obtained in this study were slightly below the minimal limit presented.

Nevertheless, it is surprising that allergic otitis externa is missing from this casuistic, as results from both the clinical presentation of cases and the treatment.

However, specific literature offers various data, sometimes contradictory, concerning the prevalence of allergic otitis externa. Thus, in a study carried out in 2007, Saridomichelakis et al. (5) noticed that 43% of otitis externa were of allergic nature, 12% were due to the foreign bodies and only 7% were parasitic. In addition, 63% were recurrent cases.

Conclusions

In dogs, otitis externa of bacterial and mycotic origin are more frequently noticed, while in cats the parasitic and bacterial ones are more prevalent.

The main clinical signs in both dogs and cats, which drew the owner's attention, were the frequent head shakings and ear pruritus.

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SEROLOGICAL SUPERVISION OF BLUETONGUE DISEASE IN THE SOUTH-EAST REGION OF ROMANIA

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Summary

In 2014, the first outbreak of Bluetongue, appeared in Buzau County in the SE of Romania. Bluetongue is a non-contagious viral disease, with vector transmission, through *Culicoides* hematophagous insects, to various species of ruminants. Due to the expansion of the area of the culicoides vectors the disease is seasonal and enzootic. In these circumstances, we have proposed, to highlight the results of the serological monitoring of Bluetongue disease in the same South-Eastern region of Romania and and in the same period in which the disease first appeared in Romania. Blood samples were taken through the Serological Screening Program of Bluetongue initiated and sustained by ANSVSA and collected samples from animals suspected of Bluetongue disease. **In 2014,** in the four counties studied: Galaţi, Brăila, Vrancea and Tulcea, 1414 blood samples were taken through the serological surveillance program and 453 blood samples were collected from animals with suspicion of disease. In this year's,the prevalence of anti-BTV antibodies in samples collected through the serological surveillance program was 1.8% in Galati and 0.45% in Braila and no positive evidence was found in sheep and goats. The prevalence of anti-BTV-specific antibodies in the samples collected from suspected animals was: in Galati was 80% (n = 5) in cattle; in Vrancea County was 86.44% (n = 236) in sheep, 89.4% (n = 179) in cattle, in Braila County was 60% (n = 5) in cattle and in Tulcea County was 25% (n = 8) in sheep and 33.33% (n = 6) in cattle. **In 2015,** through the serological surveillance program for the Bluetongue, 4528 blood samples and 51 blood samples from Bluetongue-suspected ruminants in Vrancea and Brăila counties were tested. In this year's, the prevalence of anti-BTV-specific antibodies in samples collected from suspected animals was 44,41%(n=655) of the tested cattle in Vrancea County and 6,12%(n=996) of the bovines tested in Braila County. No positive evidence was found in Galaţi and Tulcea, although both counties. Seroprevalence Atc. anti-BLV in animals with suspected disease in Vrancea County was 44.42% (n = 4) in goats, 100% (n = 1) in sheep and 100% (n = 1) in cattle. In Braila County, seroprevalence of anti-BTV antibodies was 17.17% (n = 45) in bovine suspected Bluetongue
Keywords: serological, Bluetongue, SE Romania

Bluetongue (BT) is a viral infectious non-contagious disease of domestic and wild ruminants., of variable clinical severity, characterized by mucosal inflammation, whole body haemorrhage and edema (5, 7). The etiologic agent is a *Reoviridae* family, the genus *Orbivirus* (2, 5, 7). These viruses are transmitted via

the *Culicoides* hematopoietic insects whose epidemiological importance has been proven and recognized worldwide (5, 6, 7). The bluetongue disease does not affect humans but causes economic losses through transmission to other animals. The environmental factors in Europe favored the emergence and continual expansion of the disease to the borders of Romania. In 2014, the disease was confirmed in southern Bulgaria and in three months it expanded in most areas of the country. In the same year, the first outbreak of Bluetongue in Romania in Buzau County was reported and expanded to 17 other counties, with 184 disease outbreaks identified (3, 4, 12). According to the regulations issued by the OIE, Bluetongue can only be confirmed when the presence of circulating virus is detected in both the receptive animals and the vectors in that area (8, 9, 11).

The confirmation of Bluetongue disease in Romania has had important economic consequences and financial resources are still being mobilized to compensate the owners of slaughtered animals and to prevent and control the disease as well as to permanently monitor vector populations in accordance with EC Regulation 1266/2007 (10, 14).

Materials and methods

Considering the area where the first Bluetongue outbreak was reported, we wanted to highlight the results of the monitoring of anti-BLV antibodies in domestic ruminants in Vrancea, Braila, Galati and Tulcea counties during 2014-2015 as an indicator of virus infection Bluetongue.

Blood samples were taken through the Serological Screening Program of Bluetongue initiated and sustained by ANSVSA and collected samples from animals suspected of Bluetongue disease. The veterinary epidemiological surveillance for the disease of the blue tongue was regulated by Order no.154 / 2007 approving the sanitary veterinary norms through this strategy and published in the Romanian Ministry of Health no.580 / 2007. The conditions for sampling, transport and processing of blood samples complied with the protocols contained in the Bluetongue Operations Manual, 2nd Edition 2015.

Serum samples were obtained by coagulation of the blood in the harvest tubes and corresponded to the quality required for serological (non-hemolyzed) testing. Sample processing was carried out in the Veterinary Sanitary and Food Safety Laboratory in Brăila, Galati, Vrancea and Tulcea.

Serum samples were tested by the ELISA immunoassay technique for the identification of the recombinant VP7 protein (IDEXX kit and Ingezim kit) according to the procedures of the International Standards on Organic Testing and Biological Products Manual of the International Office of Epizootics, Issue 2014, Part 2, Section 2.1 ., Cap. 2.1.3.

Results and discussion

In 2014, 1867 blood samples from domestic ruminants from the four counties studied were collected and processed (Table 1). Of these, 1537 samples came from cattle, 50 goats and 280 samples from sheep. In 2014, 1414 samples of serum were sampled and processed in the four counties, and the remaining 453 samples were taken from animals with suspected disease. From the analysis of the data obtained, the prevalence of the positive samples identified by the Serological Surveillance Program was different and much diminished compared to the prevalence of the samples taken in case of suspected disease (Table 1).

Table 1

Annual distribution of samples taken from domestic ruminants in the SE region of Romania and the results of serological tests in 2014

2014 County	Species	No. samples /serological surveillance	Results of serological tests ELISA		No. samples / suspicions of the disease	Results of serological tests ELISA		TOTAL TEST SAMPLES
			POS.	NEG.		POS.	NEG.	
Galaţi	Sheep	0	0	0	0	0	0	0
	Goats	0	0	0	0	0	0	0
	Cattle	280	5	275	5	4	1	285
Vrancea	Sheep	0	0	0	236	204	31	236
	Goats	0	0	0	0	0	0	0
	Cattle	210	0	210	179	161	18	389
Brăila	Sheep	36	0	36	0	0	0	36
	Goats	36	0	36	10	0	10	46
	Cattle	180	3	177	5	3	2	185
Tulcea	Sheep	0	0	0	8	2	6	8
	Goats	0	0	0	4	0	4	4
	Cattle	672	0	672	6	2	4	678
Total		1414	8	1406	453	376	76	1867

Seroprevalence also varied according to the species and county where the evidence comes from.

In 2014, most of the samples taken from the Surveillance Program were from cattle (n = 1342) from all counties studied: Galaţi (n = 280), Vrancea (n = 210), Brăila (n = 180), Tulcea (n = 672). Positive assays for anti-BTV antibodies were identified in Galati (1.8%) and Braila (0.45%) (Fig. 1). During this period no positive evidence was found in ovine and caprine animals monitored through the serological surveillance program.

During the same period, 453 samples of serum from animals suspected of being sick were tested. The situation was quite different, the results showing the presence of circulating BTV virus in all species in this area. Thus, in Galaţi County, 80% of the samples tested in cattle were positive, in Vrancea County, 86.44% of the samples tested in sheep and 89.4% of the samples tested in cattle were positive for the Atc. anti BTV; in Braila County, 60% of the samples tested in cattle were positive; in Tulcea county, 25% of the samples tested in sheep and 33.33% of the samples tested in cattle were positive (Figure. 2). No suspicions of goat disease have been reported.

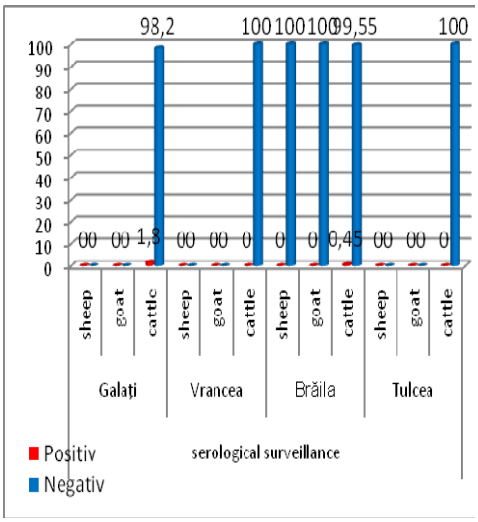

Fig. 1. Seroprevalence of Atc.anti-BLV in domestic ruminants monitored through Bluetongue Serological Surveillance Program in 2014

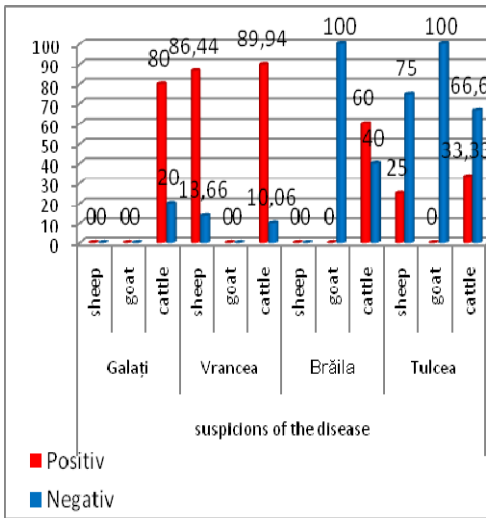

Fig. 2. Seroprevalence of Atc.anti-BLV in domestic ruminants suspected of Bluetongue disease in 2014

In 2015, 4579 blood samples from domestic ruminants from the counties studied were tested (Table 2).

From the analysis of the table, it is observed that, as in 2015, the tests were performed especially in cattle, in all counties studied: Galati (n = 1064), Vrancea (n = 656), Braila (n = 1041) and Tulcea (n = 1552). Serological testing for the identification of Atc anti-BTV in sheep (n = 262) and goats (n = 4) was performed only in Vrancea County.

Through the serological surveillance program for the blue tongue, 4528 samples of serum and 51 samples of sera from Bluetongue-suspected ruminants in Vrancea and Brăila counties were tested.

Table 2

Annual distribution of samples taken from domestic ruminants in the SE region of Romania and the results of serological tests in 2015

2015 County	Species	No. samples /serological surveillance	Results of serological tests ELISA		No. samples / suspicions of the disease	Results of serological tests ELISA		TOTAL TEST SAMPLES
			POS.	NEG.		POS.	NEG.	
Galați	Sheep	0	0	0	0	0	0	0
	Goats	0	0	0	0	0	0	0
	Cattle	1064	0	1064	0	0	0	1064
Vrancea	Sheep	261	0	261	1	1	0	262
	Goats	0	0	0	4	3	1	4
	Cattle	655	291	364	1	1	0	656
Brăila	Sheep	0	0	0	0	0	0	0
	Goats	0	0	0	0	0	0	0
	Cattle	996	61	935	45	8	37	1041
Tulcea	Sheep	0	0	0	0	0	0	0
	Goats	0	0	0	0	0	0	0
	Cattle	1552	0	1552	0	0	0	1552
Total		4528	352	4176	51	13	38	4579

From the analysis of the results obtained from the immunoenzymatic serological tests, it was revealed that through the serological surveillance program of the blue tongue, it is possible to monitor the circulation of the BTV virus in a population of susceptible animals. Laboratory analyzes have identified anti-BTV antibodies in 44,41% of the tested cattle in Vrancea County and 6,12% of the bovines tested in Braila County (Fig. 3). No positive evidence was found in Galaţi and Tulcea, although both counties are located in geographic areas with factors favoring vector hematopoietic insects.

Following tests performed on blood samples taken from suspected animals, anti-BTV antibodies were identified in all three species of ruminants. Thus, in the county of Vrancea, seroprevalence was 44.42% (n = 4) in goats, 100% (n = 1) in sheep and 100% (n = 1) in cattle. In Braila County, seroprevalence of anti-BTV antibodies was 17.17% (n = 45) in bovine suspected Bluetongue (Fig. 4).

It is evident that the serological surveillance program for Bluetongue disease is mainly carried out on serological testing of bovine animals as they play a particularly important role in the epidemiology of the disease due to prolonged viremia and the absence of clinical manifestations (13).

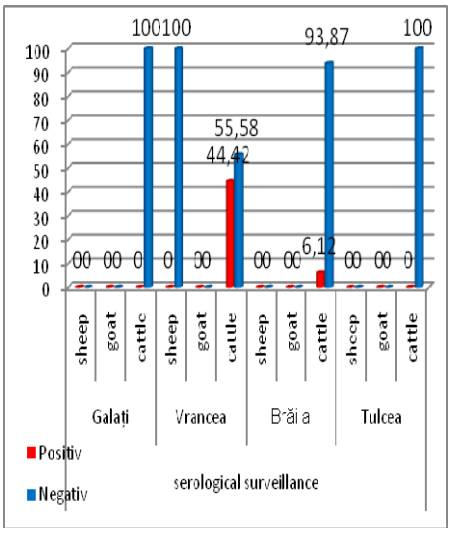

Fig. 3. Seroprevalence of Atc.anti-BLV in domestic ruminants, monitored by the Bluetongue Serological Surveillance Program in 2015

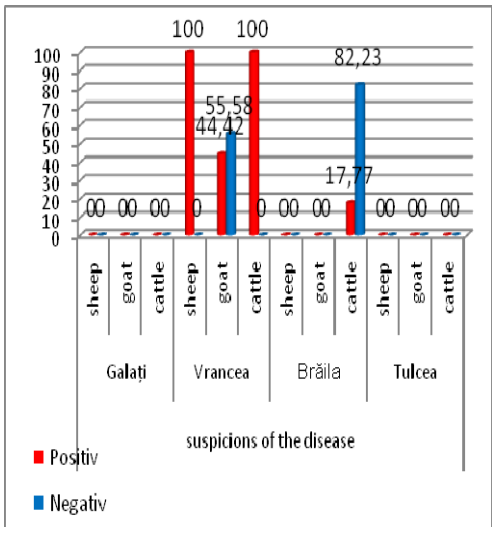

Fig. 4. Seroprevalence of Atc.anti-BLV in domestic ruminants suspected of Bluetongue disease in 2015

The comparative analysis of the data obtained in 2014 and 2015 shows the importance of serological surveillance for the identification of Bluetongue-positive animals and the Action Plan for the Prevention and Control of Bluetongue Constipation (12) The effect of these measures imposed during the two consecutive years (2014, 2015), following the identification of BTV seropositive animals, was reflected by a decrease in the number of suspected animals (from 453 animals in 2014 to 51 animals in 2015) and the geographical limitation of suspected cases of disease (2014) in all the counties studied and subsequently (2015), they were singled out only in the counties of Vrancea and Braila.

The presence of disease outbreaks in Romania plays an important role in the management of intra-Community import and trade activities with neighboring countries. The spread of the Bluetongue virus in countries in the immediate vicinity of Romania (Greece and Bulgaria) has been an important critical point, which has imposed a permanent state of alert and prepared prevention and response to this threat for a long time now.

According to the annual report of the European Union, the serotype 4 of the virus is present in Romania, which means that the circulation of ruminants both on the territory of Romania to other Member States and other Member States on the territory of Romania is carried out under Reg. (EC) 1266/2007 (10, 12, 14, 15)

Conclusions

The presence of BTV-specific antibodies in the susceptible ruminants, demonstrate that the virus is circulating in the S-E Romania and it is necessary to continue the serological surveillance of the susceptible animals simultaneously with the identification and elimination of vectors to prevent the emergence of new outbreaks of disease.

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PLANT EXTRACTS INFLUENCE THE *IN VITRO* IgG ACTIVITY DEPENDING ON THE IMMUNE HEALTH STATUS IN BOVINE

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Summary

Both enzootic leukosis and tuberculosis are recognized in bovine as major causes of economic losses by decreasing production and immune resistance in hosts. Moreover, both diseases pose health risks for other animals and humans, requiring the introduction of severe quarantine of contaminated herds or sometimes their stamping out. Thus, clarifying in detail the activities of immune effectors such as IgG, could be of use in a more rapid diagnosis and a more clear overall picture of the immune status of the animals.

The study was carried out in adult bovine (n=95), divided into three groups according to their health status: group I (n=50) - healthy, group II (n=27) - bovine enzootic leukosis virus (BELV) positive and group III (n=18) - *M. tuberculosis* (TB) reactive animals. Blood samples were collected by mammary vein puncture and the classical Mancini technique was used to quantify IgG levels. Subsequently, saline, 70° alcohol and alcoholic extracts of *Calendula officinalis* and *Echinaceea angustifolia* were added to the sera *ana partes* (50 µl/well) in a 96 well-plate, and the diffusion test was repeated using diluted sera, readings being performed after 24 and 48 h, to: a) investigate the *in vitro* activity of IgG in the presence of the plant active principles and b) to estimate the duration of the effect over time. The statistical interpretation of the data indicated that there were no significant differences between the initial values of the IgG concentrations in the sera of the three groups (28.58 ±11.52, 31.82±15.20 and 33.58±17.66 mg%, respectively). The *in vitro* treatments with the alcohol plant extracts seemed to enhance the bounding capacity of IgG, the effects being plant- and time-based. After 24 h, *E. angustifolia* showed a stronger effect in BELV animals (53.3±1.97 mg%) than in TB ones (43.17±5.47 mg%), but after 48 h, both extracts had very similar effects in groups II and III with values ranging from 63.3±3.3 mg% - *C. officinalis*, BELV group and 68.8 ±3.86 mg% - *E. angustifolia*, TB group, respectively). The results indicated that the plant extracts that were used could enhance and speed up the quantification of IgG for clarifying the immune status of the BELV and TB animals.

Keywords: IgG, bovine enzootic leucosis, tuberculosis, *E. angustifolia, C. officinalis*

Both enzootic leukosis (BELV) (1, 9, 10) and tuberculosis (TB) (8) are recognized in cattle as important causes of economic loss by decreasing production and immune resistance to hosts. In addition, both diseases present a sanitary and veterinary risk, which implies the introduction of a severe quarantine in contaminated herds, or sometimes their elimination. Thus, clarifying in detail the

mechanisms of action of immune effectors such as IgG could be useful for a faster diagnosis and a clearer picture of the immune status of animals (2, 3, 6, 13).

This study aimed at establishing the potential role of alcoholic vegetal extracts from two plants *Calendula officinalis* and *Echinacea angustifolia*, of the same (*Compositae*) family on the *in vitro* activity of bovine IgG, in order to improve the diagnostic methodology for defining the immune status of BELV and TB infected individuals.

Materials and methods

The study was conducted on adult dairy cows of Romanian Spotted breed (n = 95), divided into three groups according to their health status: group I (n = 50) - healthy, group II (n = 27), positive for infection with bovine enzootic leukosis virus (BELV) and group III (n = 17) infected with *M. tuberculosis* (TB). Identification of seroconversion to BELV was established by ELISA test (LACTELISA® BLV Ab Bi Indirect test, Zoetis) (4, 11) and the TB reactivity was monitored during the intradermal testing performed within the state surveillance program for tuberculosis on dairy farms.

Blood samples were collected by puncture of the mammary vein, allowed to clot and sera were kept at -80 □C till testing. The total IgG levels were quantified by the classical radial diffusion Mancini technique (Fig. 1, 2). For that, anti-bovine IgG serum was included in a 2% Noble agar (Oxoid). Later, serum, 70 ° alcohol and alcohol extracts of Calendula officinalis and *Echinacea angustifolia ana partes* (50 µl / well) were added to the sera in a 96-well plate, the diffusion assay being repeated with the diluted sera and readings were made after 24 and 48 hours for:

a) Investigation of in vitro activity of IgG in the presence of active principles of plants and

b) Estimation of the duration of their effect over time.

Results and discussions

IgG represents the main anti-infectious immune globulin in the bloodstream, both as proportion and as direct activity. Its concentration is lower during the primary immune response, while IgM dominates, but its level substantially increases during the secondary (booster) immune response or during permanent antigenic stimulation, such as in intracellular bacterial pathogens' presence.

Thus, quantification of the IgG concentrations could represent one of the important aims in describing the immune status in any microbial infection of the host (1, 7, 12).

The statistical interpretation of the obtained data indicated there are no significant differences between the initial values of IgG concentrations in the three experimental groups (28.58 ± 11.52, 31.82 ± 15.20 and 33.58 ± 17.66 mg%, respectively).

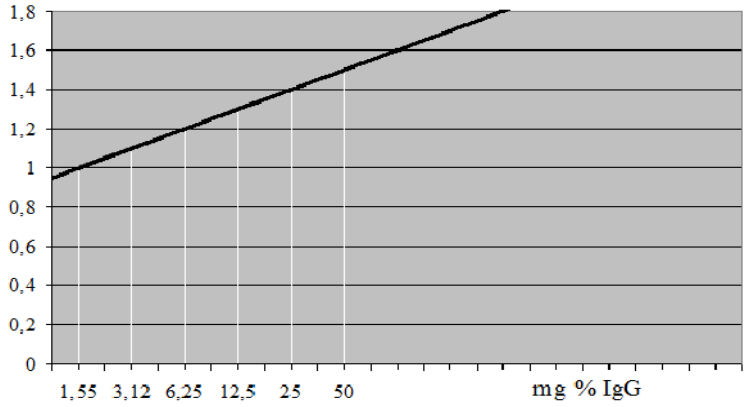

Fig. 1. Mancini test – reference curve

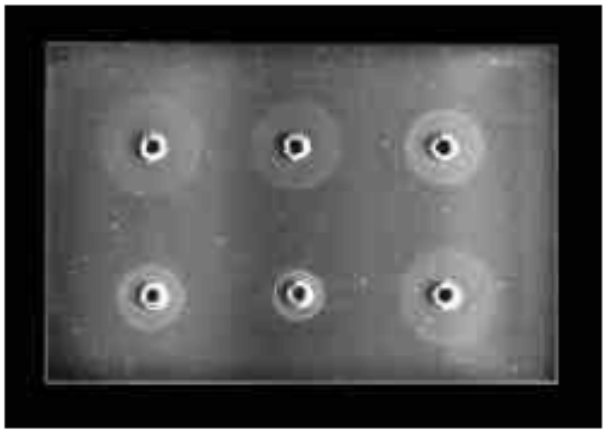

Fig. 2. Example of the dilution of sera with *C. officinalis* and *E. angustifolia* extracts for the Mancini test (anti- bovine IgG serum in agar, bovine IgG, standard, individual serum samples)

The *in vitro* treatments with alcoholic vegetal extracts seemed to enhance the binding capacity of IgG, the effects depending on the plant type and exposure time.

After 24 h, *E. angustifolia* had the strongest effect in BELV positive animals (53.3 ± 1.97 mg%) as opposed to the TB reagent ones (43.17 ± 5.47 mg).

After 48 h both extracts had similar effects in groups II and III, with values ranging from 63.3 ± 3.3 mg% - *C. officinalis*, BELV group and 68.8 ± 3.86 mg% - *E. angustifolia*, TB group.

The results indicated lower values for circulating IgG levels in healthy animals when compared to the other two tested groups (Table 1). In spite of the fact the differences between the two infected groups were not supported statistically and the expected IgG values in BELV positive animals should have been higher (5) than in the TB group, the experimental data defined a more pronounced humoral immune reactivity in the TB positive animals.

In spite of the species, similar for both groups and the same Ig type, the *Echinacea angustifolia* extract acted stimulating in BELV positive, but inhibiting in tuberculin responsive animals after 24 h (Table 2). The *Calendula* extract acted in BELV sero-converted cows more inhibiting than the *Echinacea* one in the TB positive group.

Table 1

Values of circulating IgG in the experimental groups

	Healthy cows	**BELV cows**	**TB positive cows**
Media	28.58	31.82	33.58
Deviaţia standard	11.52	15.20	17.66

Table 2

IgG values in experimental lots - pretreated sera with extracts, reading after 24 hours (mg%)

	Indicator	**Saline**	**Alc 70°**	**Calendula**	**Echinacea**
BELV	Mean	20.83	53.3	42.77	53.5
	Stdev	7.21	3.3	15.47	1.97
TB	Mean	50	54.5	52.2	43.17
	Stdev	0.06	9.81	1.91	15.47

The long term efficacy (48 h reading) was demonstrated by the increase in precipitation diameters over those of the sera treated with alcohol or saline.

In BELV positive bovine the effect of the *Calendula* extract was indifferent when compared to that of the alcohol control, while the alcoholic *Echinacea* extract preserved its stimulating effect (Table 3).

For the TB reagent group the effects were more pronounced than in the BELV group for both extracts. Both *Echinacea* and *Calendula* extracts exerted a stimulating effect in this order.

The obtained results indicated a recordable *in vitro* bioactivity of the tested extracts, but there is need for further testing of various dilution schemes for obtaining the most pronounced effect.

Table 3

IgG values in experimental groups – vegetal extract pre-treated sera, reading after 48 h (mg %)

	Indicator	Saline	Alc 70°	Calendula	Echinacea
BELV	Mean	58.86	63.3	63.3	66.63
	Stdev	1.96	0.01	3.3	3.35
TB	Mean	63.3	60	65.5	68.8
	Stdev	0.09	0.12	6.92	3,86

Conclusions

The *Calendula officinalis* extract exerted an inhibiting effect on the bovine IgG complexation activity with anti-IgG serum, while the *Echinacea augustifolia* extract acted stimulating, after 24 h, values that stand for the differentiated effect of alcoholic extracts from two plants of the same family, *Compositae*.

The efficacy of the tested extracts is also depending on the infection type (viral/bacterial) and in connection with plant species, thus 24 h the *Calendula* extract was inhibiting in BELV but stimulating in TB positive animals and the *Echinaceea* extract acted opposite. The *in vitro* exposure period lead to stimulation of the precipitation for both extracts, less for *Calendula* and more for *Echinacea* extract.

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THE ECONOMIC IMPACT OF RABIES IN THE ENDEMIC ZONES

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Summary

Worldwide, canine rabies is still responsible for more human deaths than any other zoonosis. For interrupting the transmission of canine rabies, World Health Organization (WHO) recommends vaccination of at least 70% of the total canine population and maintainig the optimal imune level for a period of 3 to 7 years, in order to eradicate this disease.

In Haiti, the canine rabies remains enzootic, with annual estimation of 130 human deaths due to dog bitings. Because 80% of the canine population is consisting in stray dogs living on the streets, parenteral vaccinations in veterinary clinics (set points) are difficult tasks. For this study, it has been used an oral vaccine consisting in a highly attenuated viral strain, SPBNGAS-HAS.

A total of 10590 dogs were vaccinated: 590 by oral pathway and 10000 by parenteral vaccination. From the total amount of vaccinated dogs, 109 were evaluated for the postvaccinal response. The 590 doses of oral rabic vaccine (ORV) were introduced in plastic capsules covered with aluminium foil and placed into a boiled pork or beef gut, in order to atract the dogs. From these, 291 baites were taken into consideration in this study: 235 (80,8%) were givent to owned dogs, 50 were placed on the street for the stray dogs (17,2%) and for the 6 (2%) of the baites, the location wasn't registered. 283 dogs (97.2%) accepted the baits and the operators reported that 272 baites were perforated (93.7%), suggesting exposure to the vaccine. From the baits taken into consideration, 277 (95.2%) were ingested by the dogs or recovered and only 14 baits (4.8%) were not recovered.

Keywords: rabies, oral vaccination, economic impact

Worldwide, canine rabies is still responsible for more human deaths than any other zoonosis (5, 7).

Providing post exposure prophylaxis (PEP) for humans bitten by suspected dogs is a necessity, but vaccination of the reservoir species (mainly dogs) is known to be the most cost – effective solution in fighting against rabies (12).

For interrupting the transmission of canine rabies, World Health Organization (WHO) recommends vaccination of at least 70% of the total canine population and maintaining the optimal immune level for a period of 3 to 7 years, in order to eradicate this disease (16).

Many developed countries succeeded to eradicate rabies by parenteral vaccination in veterinary clinics (set points), but very few countries with medium or low incomes reached this target.

In poor developed countries, many barriers have hampered effective parenteral vaccination, including lack of funds, infrastructure and political will, poorly organized campaigns, the inability of the owners to restrain their dogs, the inability of operators to approach to the dogs without extraordinary effort, and the large number of stray dogs (8).

For the stray dog populations, the oral vaccination is considered a good option (16). Even if it was used in some areas (2), this method is not yet widely integrated into existing vaccination campaigns.

Materials and methods

In Haiti, the canine rabies remains enzootic, with annual estimation of 130 human deaths due to dog biting (13, 14). The barriers hamping rabies eradication include economical, educational and cultural factors (10). Moreover, because 80% of the canine population is consisting in stray dogs living on the streets, parenteral vaccinations in veterinary clinics (set points) are not easy to perform. Even if the constant effort of the Haitian Ministry of Agriculture, Natural Resources and Rural Development (MANRRD) lead to an improvement in the number of rabic vaccination in set points, yet through national vaccination program is covered only 50% of the local dog population.

For this study, it has been used an oral vaccine consisting in a highly attenuated viral strain, SPBNGAS-HAS, which derives from SAD L16, a DNA clone of the strain used for SAD B19 oral vaccine. It was obtained by performing two mutations of the amino acids in the positions 194 and 333 and by adding an additional gene for a same glycoprotein, in order to enhance the safety profile compared to other oral vaccines (4, 11).

In 2016, the USA Center for Disease Control and Prevention (CDC) assessed a rabies vaccination campaign conducted by MANRRD in partnership with Christian Veterinary Mission, Humane Society International and IDT Biologics in Croix-des-Bouquets, Haiti to find solutions for eradicating rabies at national level. A number of 10590 dogs were vaccinated using the following methods: set point vaccination (veterinary clinics), door-to-door vaccination, oral vaccination (ORV) and capturing the dogs, followed by vaccination and release (6).

From the total amount of vaccinated dogs, 109 were evaluated for the post vaccinal response after taking blood samples (1.5 – 3 ml). The dogs were divided into two groups, consisting in 50 parenteral vaccinated dogs and 59 oral vaccinated ones. The size of the groups was determined by the Fleiss method, with alpha=0.05 and beta=80%, with a 5% error for the parenteral vaccine and 25% for the oral one. This study was conducted in accordance with the CDC 2757DOTMULX protocol, approved by MANRRD.

In the firs place, it were used 7000 Rabvac vaccine doses (batch: 4130242A, with shelf life until 08.09.2017, produced by Boehringer Ingelheim Vetmedica, Saint Joseph, MO, USA and provided by CDC), which ended on day 11 of the 14

campaign days. Then, MANRRD decided to use 3000 doses, which expired five months before, Rabisin R (batch: L399308, with shelf life until 20.03.2016, Leon, France).

The 590 doses of ORV (experimental) were provided by IDT-Biologika, Dessau-Rosslau, Germany (Vaccine strain: SPBNGAS-GAS, batch: 0010716, expiring in October 2016). The vaccine was introduced in plastic capsules covered with aluminum foil and placed into a boiled pork or beef gut, in order to attract the dogs.

Prior to the distribution of the oral vaccine, a safe review was carried out by CDC, MANRRD and IDT Biologika, in accordance with WHO recommendations (16). During the campaign, the owners consented for the vaccination by the ORV method. A telephone number was available for the owners to report possible adverse effects or exposure of persons by contact with the dog's vaccine or saliva in the first 48 hours post-vaccination. At the end of each day, operators assured that no vaccine capsules remained in the environment.

A capsule bite surveillance system was used during the vaccination, and one week after the campaign, data were obtained. The information targeted the dog's response after catching the bait, perforation of the capsule and the operator's ability to collect the capsule when it was not ingested by the dog.

The Rabvac 1 vaccine has arrived in two tranches (3500 vaccines each); the first tranche was kept at 4-8°C, but the second one was kept at ambient temperature (assumed duration of 2-4 days) and properly stored after, during the week before the campaign. Prior to use, the titre of antigens was measured in all three types of vaccine: the improper stored one, the expired one and the properly stored one and proven to be identical in all.

For the detection of prevaccinal neutralizing antibodies (rVNA), there were used serum samples and the rapid fluorescent focus inhibition test (RFFIT), according to the standard protocol (Yager ML, Moore SM, the rapid fluorescent focus inhibition test). It was considered positive an RFFIT> 0.005 IU/ml (15).

For testing the presence of antibodies in ORV dogs, it was used the blocking ELISA (O.K. Servis BioPro, PRague, Czech Republic) according to the standard protocol. It was considered positive for values higher than 40%.

The parenteral vaccinated dogs chosen for postvaccinal antibody detection were divided in three groups: 1) vaccinated with proper stored vaccine (21), 2) vaccinated with unproper stored vaccine (20) and 3) vaccinated with expired vaccine (18).

There was conducted an interview with the owners whose dogs had pre-vaccinal rabies virus neutralizing antibodies (RVNA), which showed that unvaccinated animals participated in battles with other dogs, had unexplained injuries and spent a lot of time alone on the street.

Results and discussion

From the 590 baits containing ORV, 291 baites were taken into consideration in this study (table 1): 235 (80,8%) were givent to owned dogs, 50 were placed on the street for the stray dogs (17,2%) and for the 6 (2%) of the baites, the location wasn't registered. 283 dogs (97.2%) accepted the baits and the operators reported that 272 baites were perforated (93.7%), suggesting exposure to the vaccine. From the baits taken into consideration, 277 (95.2%) were ingested by the dogs or recovered and only 14 baits (4.8%) were not recovered (table 1).

Table 1

Dogs reaction at the baits and ORV capsule

Accepted baits	Refused baits		Accepted baits									
Exposure to the capsule	None		Perforated capsule, not ingested		Perforated capsule, ingested		Unperforated capsule		Unknown		TOTAL	
Place where the baits were given	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Private property	8	3.4	120	51.1	100	42.6	7	3.0	0	0.0	235	80.8
Street	0	0.0	29	58.0	17	34.0	0	0.0	4	8.0	50	17.2
Unknown	0	0.0	3	50.0	3	50.0	0	0.0	0	0.0	6	2.0
TOTAL	8	2.7	152	52.2	120	41.2	7	2.4	4	1.4	291	100

The dogs that had pre-vaccinal rabies virus neutralizing antibodies (RVNA) (10), were not included in this study: 8 parenteral vaccinated dogs and 2 oral vaccinated. Therefore, 44/48 (92.7%) of the parenteral vaccinated dogs had detectable RVNA (>0.05 IU/mL) with a GMT of 1.3 IU/mL, and 34/57 (59.3%) of the oral vaccinated dogs had detectable RVNA (> 0.005 IU/mL, $p<0.1$) with a GMT of 0.5 IU/mL. When antibodies were measured using blocking ELISA, 44 dogs (77.8%) had detectable antibodies (> 40% blocking, $p <0.05$).

Dogs receiving parenteral vaccine, irrespective of the storage conditions (appropriate or inadequate) had the same antibody responses; 100% and 81.3%, respectively (>0.05 IU/mL, $p>0.05$). The GMT for dogs that received the properly stored, unproperly stored and expired (adjuvanted) vaccine were 1.2 IU/mL, 0.8 IU/mL and 5.5 IU/mL respectively, values similar to those of Lankester et al., which allows us to argue that these high-quality vaccines can be used even if stored at unproper temperatures and they maintain their immunological capacity (9). However, every vaccinal product that has been unproperly stored at certain moment, must be evaluated before using.

No death was reported among the 590 dogs that received the bait and no call to announce human exposure to the bait or vaccine. Moreover, out of 32 dog bites reported in the vaccination area during the campaign and two weeks post-vaccination, none were caused by the dogs that were vaccinated orally. Manual administration of the ORV was crucial to reduce exposure among community members, since only 14 (4.8%) vaccine capsules were left in the community intentionally. This information supports the fact that in Haiti, using the manual administration of SPBNGAS-GAS vaccine packed in bowels as a vaccination method, the likelihood of exposure of humans and other animals than dogs to ORV is low.

The success of oral vaccination is complex and requires a number of critical steps: the animal must be attracted by the bait, perforation of the capsule, the vaccine must have a proper contact time with the oral mucosa. Any lack in this protocol can lead to failure. The SPBNGAS-GAS vaccine, embedded in a piece of gut, had an appealing effect on dogs (97.2% acceptance of bait). Recent studies in dogs and wolves, focusing on this critical step, found acceptance of the bait varying between 47% and 93% (11). Acceptance of the bait in Philippines using identical baits and capsule was similar (96.1%) (3). Furthermore, operators observed that 93.4% of dogs perforated the capsule, indicating exposure to the vaccine. Starvation in the dog population in Haiti is likely to contribute to this high percentage of acceptance of bait.

Overall, 77.8% of the oral vaccinated dogs had post-vaccine anti-rabies antibodies. The high rate of antibody production among these dogs in Haiti suggests that SPBNGAS-GAS vaccine can be used to improve the coverage rate in hardly accessible dog populations where parenteral vaccination failed to reach the desired level of coverage (1).

From an economic point of view, the two weeks campaign cost $ 52 840, of which: $20 000 for the flight tickets of the the CDC and USDA staff; $ 15 400 for the work teams (table 2); $11 470 for materials (table 3) and $5 920 for food and personal requirements.

Table 2

Staff costs

Staff	Number	Cost per piece	Days	Total costs
Surveillance team (3 persons CVM)	6	25.00	12	1 800
Dogs counting team (3 persons CVM)	6	25.00	12	1 800
Vaccination team – set point	18	25.00	12	5 400
Vaccination team – door to door	9	25.00	12	2 700
Capture, vaccination and release team	9	25.00	12	2 700
Blood sampling team	1	25.00	4	100
Promotion team	3	50.00	6	900
				$15 400

Table 3

Material costs

Materials	Number	Cost per piece $	Total costs $
Needles / syringes	8 000	0.04	320
Thermal-insulating boxes	10	40.00	400
Educational materials	750	1.00	750
GPS devices	6	500.00	3 000
Manual/mechanical counterings	12	20.00	240
Formulary	1 000	0.10	100
Collars	9 000	0.09	810
Pens / Mini tablets	150	1.00	150
Materiale pentru prelevarea sângelui	250	1.00	250
Water pistols	10	50.00	500
Ink	10	10.00	100
Safety gloves	10	90.00	900
Butterfly nets	6	150.00	900
Dog nets	6	100.00	600
Material control	1 000	1.00	1 000
Fuel	1	1,500.00	1 500
		Total	**11 470**

Conclusions

Recent and numerous vaccination programs have managed to control rabies in settlements having poor economic resources, however, these programs have not achieved sufficient and sustainable coverage to eliminate canine rabies at national level.

The success of rabies elimination programs in poor countries has been constrained by procurement and distribution logistics as well as trained staff.

In countries were rabies is enzootic, there are also very many stray dogs, which make more difficult the vaccination strategies.

Vaccinating in veterinary clinics (set points), using parenteral vaccines, can be difficult to achieve in settlements having poor logistics and inaccessible canine populations.

Oral vaccination can overcome some of these limitations.

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SEROPREVALENCE OF LEPTOSPIROSIS IN SOME FARMS OF ALGIERS (ALGERIA)

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Summary

Although animal leptospirosis is an important zoonotic disease it remains underestimated in Algeria. In order to assess its spread in animals, 140 samples (100 sera, 20 urines and 20 kidneys) were collected from four mixed farms. All serum samples were screened using the standard micro-agglutination test (MAT) while urine and kidneys were tested for presence of bacteria for rats. With MAT, the overall prevalence was 30.43 % (14/46), 4.16 % (1/24), 20 % (2/10) and 10 % (1/10) respectively in cattle, sheep, dogs and rats. The predominant serovar was Hardjo in cattle (8/46). The presence of circulating antibodies in cattle suggests a natural exposure to Leptospira spp. implementing appropriate cattle vaccination programs would be a good step forward.

Keywords: Leptosirosis, serology, cattle, sheep, dogs.

Leptospirosis is an emerging zoonosis (4, 9) with a worldwide distribution (25). The pathogenic agents of leptospirosis are bacteria from the genus Leptospira; including Leptospira interrogans which contains pathogenic serovars (17).

About 250 pathogenic serovars are known and represented in 24 antigenically related serogroups (6, 34).

The World Health Organization (33) estimates incidence of the disease is higher in impoverished populations in developing countries. Animal leptospirosis has been reported in Algeria since 1950 (13). However, there is minimal epidemiological data on farms and so the extent of leptospirosis is unknown. Leptospira spp. can persists in wet environment for weeks (5). Some domestic and wild mammals such as rodents act as maintenance hosts by carrying specific Leptospira serogroups (7, 26).

Leptospirosis is zoonotic disease, that is why it is real public health threat (23). Furthermore, leptospirosis induces economic losses caused by reproductive disorders in cattle herds (28, 32). Pets can be vaccinated.

In Algeria, very few studies have been undertaken on leptospirosis. The aim of this study was to assess the extent of leptospirosis in cattle, sheep, dogs and rats in farms with a history of presumptive leptospirosis as infertility or sub icteric and to determine which Leptospira serovars are present.

Materials and methods

Algeria has a Mediterranean climate with warm summers and mild winters, and rain with an average of 180 millimeters rainfall per year, mostly between October and April (8).

The average temperatures hover around 11°C-12°C in January, while in August (the warmest month) the recorded temperatures are often 25°C-26°C. Summer is sunny, but there is always marked humidity.

From January 2015 to December 2015, four farms containing mixed animal species, and all in the surrounding environs of Algiers were identified for study, based on the following criteria: clinical signs of ruminant leptospirosis (abortion, infertility or marked icterus), multiple animal species in the same pastures (cattle, sheep and dogs) and lack of vaccination against leptospirosis (Table 1). one hundred blood samples from cattle, sheep, dogs and rats were collected aseptically (Table 1). The serum was centrifuged at 800 g for 10 minutes and stored at -20°C until analyzed.

Table 1

Description of blood sample sizes across farm and species

Animal	Farm 1	Farm 2	Farm 3	Farm 4	Total
Cattle	12	12	8	14	46
Sheep	6	7	5	6	24
Dog	2	3	3	2	10
Rat	5	5	4	6	20

In every farm, rat traps were placed in field sites, with fresh tomatoes as bait. Captured 20 rats were transported to the Higher National Veterinary School for euthanasia and necropsy. Rat blood was collected by intracardiac venipuncture and centrifuged as the precedents.

Animals were euthanized, and kidneys and urine aseptically collected and transported under cold to the Institute Pasteur of Algiers. Cultures were performed using Ellinghausen McCullough Johnson Harris (EMJH) broth medium (3–4 tubes/sample) (15). Renal tissue was inoculated into culture media while only 2–3 drops of urine (diluted at 1% in EMJH) were inoculated into the medium. All cultures were incubated at 28–30°C for up to 13 weeks.

Cultures were examined weekly by dark-field microscopy to detect growth of Leptospira. The result was recorded as “negative”, if there were no visible leptospiras.

All serum samples were examined by the standard micro-agglutination test (MAT) (11) at the Institute Pasteur of Algiers.

The MAT was performed using the following antigens provided by the French National Reference Center for Leptospirosis (Institute Pasteur of Paris):

Australis (strain Australis), Autumnalis (strain Autumnalis), Bataviae (strain Bataviae), Canicola (strain Canicola), Ballum (strain Castellonis), Cynopteri (strain Cynopteri), Grippotyphosa (strain Grippotyphosa), Sejroe (strains Hardjo), Hebdomadis (strain Hebdomadis), Icterohaemorrhagiae (strains Icterohaemorrhagiae and Verdun), Panama (strain Panama), Pomona (strain Pomona), Pyrogenes (strain Pyrogenes), Tarassovi (strain Tarassovi) Celledoni (strain Celledoni), Djasiman (strain Djasiman), Mini (strain Mini), Sarmin (strain Sarmin), Shermani (strain Shermani), Javanica (strain Javanica) and Louisiana (Louisiana). A non-pathogen serogroup Semaranga (strain Patoc) was also used.

Serum samples were screened, and positive serum were titrated to the end-point using standard methods. Serum samples were screened at a dilution of 1/ 100 to 1/ 3200 for sheeps and cattles, 1/ 40 to 1/ 1280 for rats and 1/ 80 to 1/ 2560; for dogs. Positive serums were titrated to the end-point using standard methods

Results were considered positive when 50 % or more leptospiras was agglutinated. Two standard serum controls (positive and negative) were conducted each time. The positive samples were identified and titrated realizing serial dilutions in order to obtain the final antibodies titer. Seropositivity was determined based on the animal species; the sample was considered positive when titers was 1:100 for ruminants and 1:40 for dogs and rodents (27).

Results and discussions

The total seroprevalence in cattle, sheep, dogs and rats, the occurrence was of 30.43 %, 4.16 %, 20 % and 10 % respectively (Table 2).

Table 2

Seroprevalences and highest microscopic agglutination test (MAT) titers against presumptive Leptospira serogroups collected in mixed farms of Algiers

Animal	Farm 1	Farm 2	Farm 3	Farm 4	Positive MAT / Total sera
Cattle	6/12 (50%) 3=Hardjo 1:200/1:200/1:400 1=Icterohaemorrhagiae 1:800 3=Pomona 1:800 1=Canicola 1:800 1=Grippotyphosa 1:800 1=Australis 1:800	3/12 (25%) 2=Hardjo 1:400/1:400 2=Castellonis 1:400/1:200 1=Australis 1:1600	2/8 (25%) 1=Grippotyphosa 1:1600 1=Patoc 1:400 1=Castellonis 1:800	3/14 (21.42%) 3 Hardjo 1:400/1:400/1:200 1= Icterohaemorrhagiae 1:3200 1=Castellonis1:1600 1=Patoc 1 :200	14/46 (30.43 %)
Sheep	1/6 Icterohaemorrhagiae 1:1600	0/7	0/5	0/6	1/24 (4.16 %)
Dog	0/2	1/3 Australis 1 :5120	1/3 Canicola1: 2560	0/2	2/10 (20 %)
Rat	1/5 Grippotyphosa 1:40	0/5	1/4 Australis 1: 160	0/6	2/20 (10%)

In cattle, each farm showed at least one animal seropositive then, leptospirosis seroprevalence varied from 10.71 % to 32 %. In positive animals, antibody titers ranged from 1:200 to 1:3200 (higher titrate observed with serovar Icterohaemorrhagiae).

Among the 14 positive sera, four reacted to only one serogroup, seven showed cross-reactions in which one serogroup predominated, and three reacted with two or more serogroups at the same degree. Our results showed that Leptospira interrogans serogroup Hardjo was the most frequently encountered (8/46) followed by Castellonis (4/46).

In sheep, only one sample of 24 tested was found positive, and it was against Icterohaemorrhagiae (1:1600) while in dogs, two of 10 dogs tested were found seropositive (Australis 1:5120 and Canicola 1:2560).

All captured rodents (24) were identified as Rattus norvegicus.

In the rats, MAT-positive was defined by a titer 1:40. Two samples were positive (Grippotyphosa 1:40 and Australis 1:160). However, culture was negative for all of them.

The major tests used in diagnostic of leptospirosis are MAT, molecular tests and culture. These tests have low sensitivity and the latter two can be problematic because of the expense required (molecular tests) and the length of time to gain a result (culture). For this reason, MAT is considered the reference serological test (16; 24).

In this study, which was conducted to estimate the extent of Leptospira spp. infection in animal populations belonging to some mixed farms of Algiers, Leptospira interrogans serogroup Hardjo was most frequently recorded in cattle (8/46, 17.39%) similar to other works reporting serovar Hardjo as the main cause of leptospirosis infection among cattle (18).

The results presented here, showed the importance of leptospirosis as a possible cause of bovine infertility problems in dairy farms area. Therefore, the clinical manifestation of Leptospira spp. serovar Hardjo infection in dairy cattle may be a notable cause of economic losses in the livestock industry because of infertility, agalactiae infections and abortion (1, 20).

As the vaccination of cattle against leptospirosis is not implemented in Algeria, the presence of circulating antibodies in cattle suggests a natural exposure to Leptospira spp. serovar Hardjo.

In sheep, the seroprevalence was (4.16 %) close to that reported in Thailand by Suwancharoen et al. (31). On the other hand, higher seroprevalences were recorded in other countries (25, 26) in relation to the animals' water source.

Seroprevalence in dogs (20 %) was similar to that reported by Kikuti et al. (19). Dogs are considered maintenance hosts for serovar Canicola (35;26), and so the one cow reacting positively to this serovar suggests that dogs play a role in cattle infection and therefore cross-infection occurs between cattle and non-vaccinated dogs (35).

In order to control spread of leptospira infection in dairy cattle, it is highly advised to generalize dog vaccination and prevent stray dogs as well as wild carnivorous animals from accessing the farms and cattle pens.

The reasons that serum from an animal reacted with various serovars could be a cross-reaction among various serovars or infection with more than one serovar. However, it was recommended that the serovar providing highest antibody titer could be an infecting serovar (10).

In rats, the seroprevalence was higher than that recorded (16.5 %) in Trinidad (30) depending mainly on risk factors. Leptospira serovars in rats could be a source of infection to humans (22).

The study highlights that the main cause of leptospirosis among cattle was probably Leptospira spp. serovar Hardjo and suggests that serogroups could have been circulating within the animal populations, which highlights the importance of leptospirosis as a possible cause of bovine infertility problems in the Algiers dairy farms area.

Conclusions

Specific host-serovar combinations seem to be widely spread: Rattus species and serovar icterohaemorrhagiae (2), mice and serovar Ballum (29), pigs and serovar Pomona (3) while a cattle was revealed seropositive against L. canicola and one sheep against L. icterohaemorrhagiae.

Only two rats from the 20 tested were seropositives against Grippotyphosa and Australis, however bacteriology was negative.

As the vaccination of cattle against leptospirosis is not implemented in Algeria, the presence of circulating antibodies in cattle suggests a natural exposure to Leptospira spp. serovar Hardjo.

In order to control leptospirosis and to reduce the risk of reproductive loss, enforcing safety measures seems highly recommended. Measures such as preventing cattle from having close contact with other reservoirs like dogs, rodents, pigs and ruminants, and implementing appropriate cattle vaccination programs would be a good step forward.

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