UNIVERSITY OF THESSALY

SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY MEDICINE

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

LABORATORY DIAGNOSTIC APPROACH TO CANINE INFECTIOUS DISEASES OF WILDLIFE AND PUBLIC HEALTH IMPORTANCE

A thesis submitted for the degree of Doctor of Philosophy

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Επιπλέον, απευθύνω ευχαριστίες στην καθηγήτρια κα Αγγελική Ρόδη-Μπουριέλ για τη συμβολή της στις μεταπτυχιακές μου σπουδές και την περαιτέρω εξοικείωσή μου με τις τεχνικές της μικροβιολογίας. Επίσης, θα ήθελα να εκφράσω τις ευχαριστίες μου στην καθηγήτρια κα Ζωή Πολυζοπούλου για το ενδιαφέρον της κατά την πορεία της διδακτορικής μου διατριβής και τις παρατηρήσεις της κατά τη διόρθωση ερευνητικών εργασιών. Ευχαριστώ την καθηγήτρια κα Ευθυμία Πετεινάκη για το ενδιαφέρον της, ειδικά κατά το στάδιο της ολοκλήρωσης της διατριβής.

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CHAPTER I

1. INTRODUCTION

1.1 Dog

a) Dog and their role in human life and society

Dogs play an important role in modern society, enhancing the psychological and physiological well-being of many people. Interactions between dogs and humans are thought to begin at least 18,000 years ago (Thalmann et al. 2013), and they were the first species of animal to be domesticated, towards the end of the last glacial period (Clutton – Brock 1995). Domesticating is a coevolutionary procedure in which a population of an animal species reacts to selective pressure while adapting to a new niche including another species with evolving behaviors (Freedman 2014). Ancient DNA indicates that dog domestication preceded the cultivation of lands (Vila 1997, Thalmann 2013) and it seems that dogs were domesticated by hunter-gatherers and not farmers (Schleidt 1998). The dog–wolf divergence time was also estimated to be 36,900–41,500 years ago. As domestication must have occurred subsequent to the dog–wolf divergence and before Southeast Asian dog divergence (∼17,500–23,900 years ago), it seems that the onset of dog domestication lies between ∼20,000 and 40,000 years ago.

It is probable that the first species to be domesticated would have certain characteristics; a flexible diet (non-totally meat based), running and hunting ability to provide food, suitable size to be controlled by humans, right temperament (Wang et al. 2008). These traits suggest the family *Canidae*, as wolves are one of the most gregarious, sociable and cooperative animal species (Schleidt and Shalter 2003, de Waal 2006). Also, there is evidence of convergent evolution between dogs and humans, as it is demonstrated by a DNA sequencing study in 2013, which concerns the genes for digestion, metabolism, neurological processes and cancer and may be attributed to common selection pressures (Wang 2013). In this way, dogs have evolved skills for understanding human social and communicative behavior, superior to those of other mammals more closely related to humans phylogenetically (chimpanzees, great apes). For example, dogs can learn the name of over 1000 objects and follow human gestures.

It is not clear how the procedures of domestication took place and a 2002 study suggested that human ancestors and wolves domesticated each other, as a form of alliance which resulted in modern humans and dogs (Taçon and Pardoe 2002) bringing many changes in human psychology, hunting practices and social behavior. However, through its domestication, dogs were gradually integrated in the natural environment of humans and thus established relationships and contacts in these new groups, composed by different species (Miklosi et al. 2004). They were used as hunting tools, warning system, guardians of herds, carriers of loads and companions. In this way, dogs are in continuous interaction with human, wildlife species as well as other domesticated animal species.

Nowadays dogs, apart from being pets or living as feral or village animals, are used in various contexts. They live and work with humans in many roles, such as herding and guarding livestock, hunting, guarding property, pulling loads, detection, assisting police and military. Also, they are used to aid humans with physical or mental disabilities in various situations; guide dogs, hearing dogs, psychological therapy dogs, service dogs (wheelchair assistant for mobility, epilepsy monitors of seizures, aid for families with autistic children, hypoglycemic detectors for diabetes). The role of these assistance dogs is of increasing interest in U.S.A. where the state is trying to provide equal accommodation for people with disabilities and allow them to have full public access with the animals (Hart and Yakamoto 2017). In general, dogs participating in activities by helping out humans are defined as working dogs.

The population of dogs has been increased and expanded around the globe as human population has grown but they are not treated in the same way in all societies or even in a single community. For example, a companion dog may be kept indoors permanently or transferred in a handbag while a working dog may be constantly living in a yard. Consequently, there are distinct types of dogs: owned dogs regarded as members of the family or valued for their work and their movements are controlled, free-roaming dogs (including owned or stray) depending partly on humans for food or shelter and dogs avoiding human contact, characterized as feral (Hughes and Macdonald 2013). One distinct particular type is represented by the dingo which has been wild for thousands of years (Macpherson 2005). These distinct groups of dogs exhibit different behavioral patterns resulting in positive and negative interactions with human and wildlife populations.

The variety of roles of dogs throughout the globe has led to their exposure to pathogens and their involvement in the transmission of over 60 zoonotic infections (Baxter and Leck 1984). Also, these extensive differences of their uses make a great difference in their role in transmission of zoonoses and the ability of humans to prevent them. Apart from that, climate change and alteration of ecosystems by human activities (deforestation, habitat fragmentation, pollution, climate change, urbanization) have impact on the transmission of pathogens from animals (including dogs) to humans and vice versa (Daszak et al. 2000, Cunningham et al. 2003, Cunningham and Daszak 2003). Also, there is increased proximity among human populations, domestic animals as dogs and wildlife species due to transformation of habitats and human activities (Daszak et al. 2001, Dobson and Foufopoulos 2001, Wolfe et al. 2007), probably leading to spill-over from one reservoir to another and to the environment.

b) Statistics about dog population and ownership

The current population of dogs is estimated to be between 700 and 900 million according to recent studies (Hughes and Macdonald 2013, Gompper 2014). There are divergent reports of the ratio of dogs to humans across the world. For instance, reported ratio can vary from 91 dogs/100 people in the Philippines (Beran 1982) to that of 6.7dogs/100 people is referred in South Africa (Otranto et al. 2017). More often, the referred ratio for the majority of population studies varies from 10-33 dogs/100 humans (Davlin and Vonville 2012). Nowadays, dog population management represents a goal of modern societies, as humans try to preserve the population size of dogs in a desirable proportion to their population and needs. In this way, they make efforts to record the density of dogs, to control the number of unwanted or stray dogs and reduce the risks for Public Health provoked by this animal species (Hiby et al. 2017). However, there are very few studies describing the impact of dog population management intervention in the current literature.

In general, 361 million dogs considered to exist in Asia, 102 million in Latin America and Carribean, 88 million in Africa, 74 million in Europe and 73 million in North America (Otranto et al. 2017). 67 million and 118 million dogs live in U.S.A. and India respectively (Hughes and Macdonald 2013, Wallace et al. 2017). Approximately 50% of the households in developed nations own a pet which may be or not be a dog

(Westgarth et al. 2010), while the population of stray dogs is estimated to be 200 million, according to World Health Organization. Dog remains one of the most popular owned pets (Westgarth et al. 2013).

In a recent study, the percentage of UK households owning a dog is 30% (Murray et al. 2015) which means that the population of pet dogs is consisted of 11 million animals. The population of pet dog owners in Ireland in 2006 was calculated to be 640.620 and the density of these households was higher in urban areas (Downes et al. 2009).

Concerning European Union, Germany ranked first with a dog population of approximately 8.6 million in 2016, followed by the United Kingdom (UK) with 8.5 million. The number of dogs in Europe has increased year over year since 2010, with the number of dogs significantly increasing by six million from 2012 to 2014. The total amount of dogs reported in 2014 is estimated at 81 million.

In 2017, a total of about 89.7 million dogs lived in households in the United States as pets. In comparison, some 68 million dogs were owned in the United States in 2000.

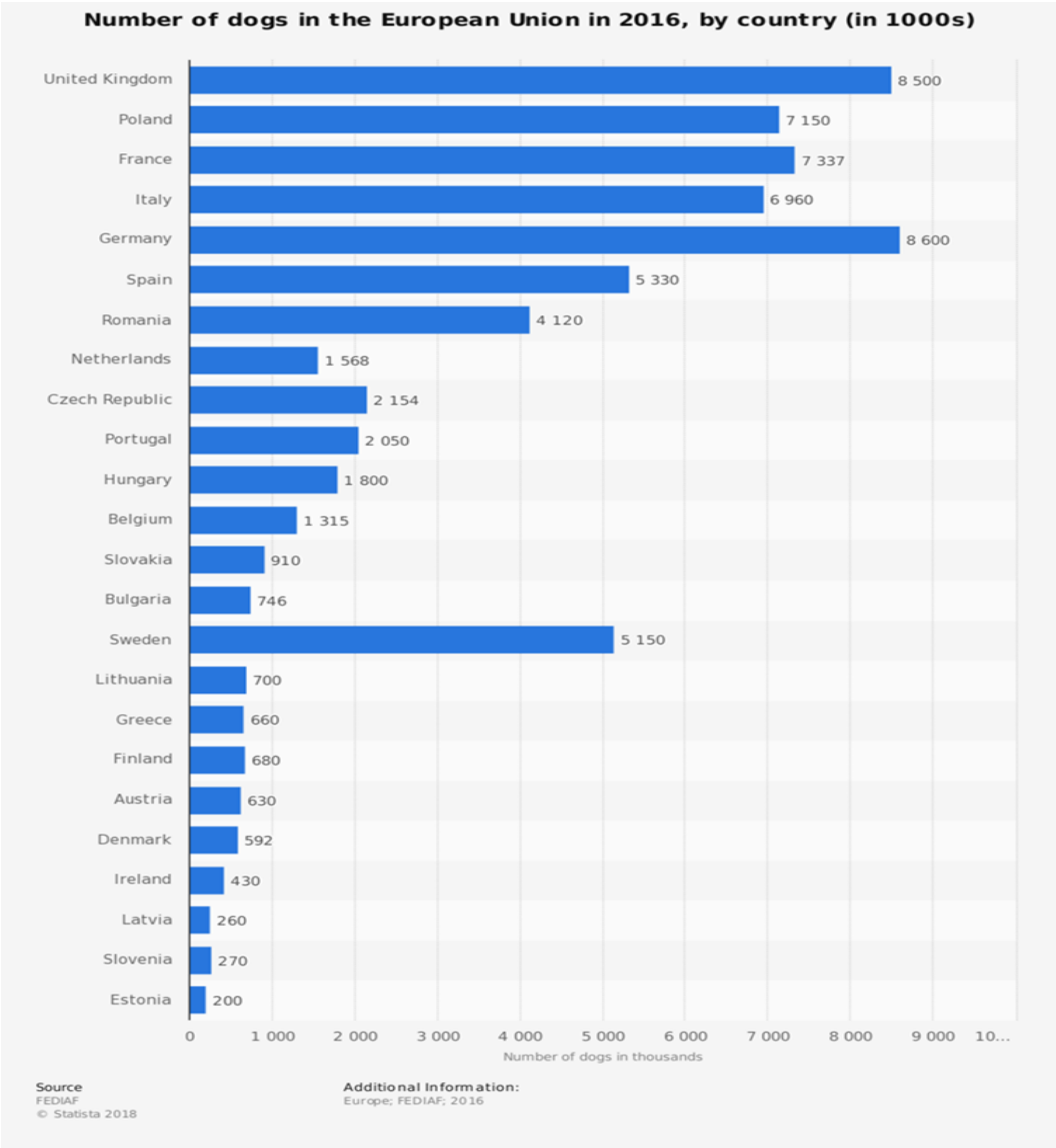

Figure 1. Number of pet dogs in the European Union by country, as of 2016.

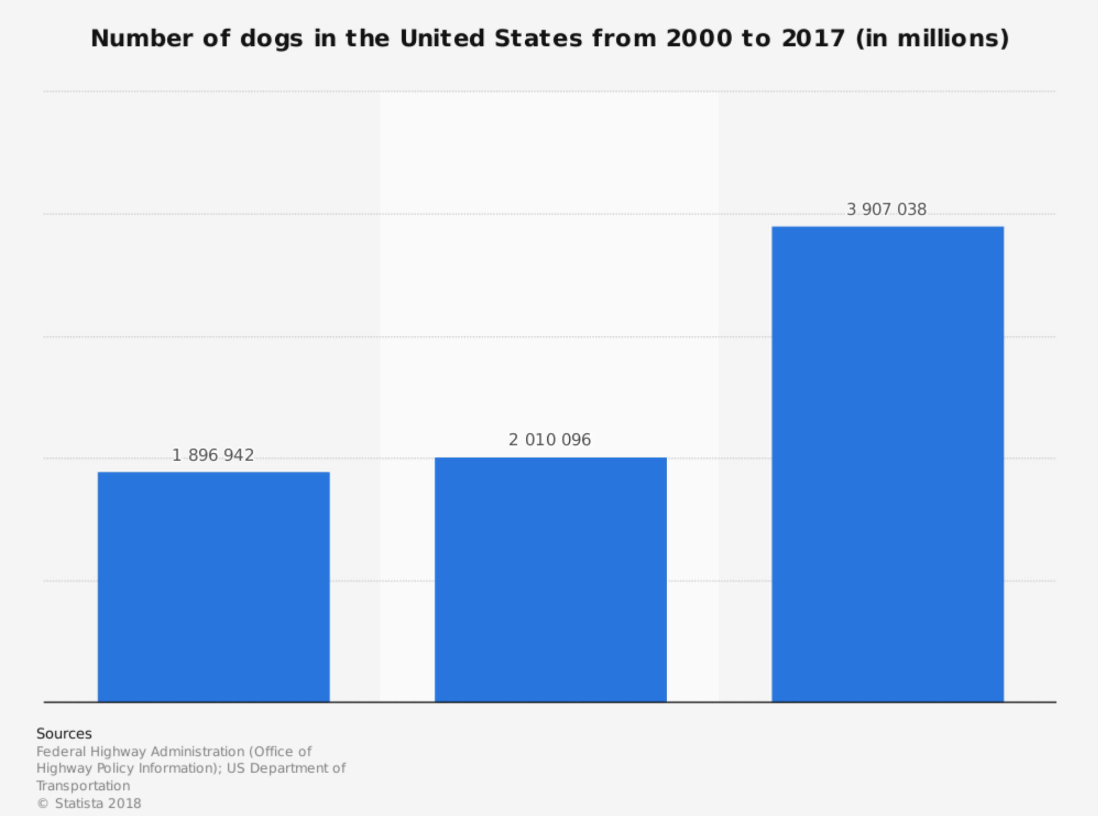

Figure 2. Number of dogs in the United States from 2000 to 2017.

c) Physical and psychological health advantages from dog ownership

In general, people own dogs for many different reasons (companionship, sport, entertainment, work-assistance) and pet ownership is an established feature of every human culture (Sterneberg van der Maaten et al. 2016). The knowledge that interaction with animals contributes to well-being and physical health of humans is not new and several studies have been conducted towards this direction.

Firstly, in the 1980s Friedmann published a longitudinal study stating that patients with coronary disease who owned a pet had one third of the mortality rate a year after discharge than those who did not. This fact led to increasing use of human-animal interaction in health research and practice.

Dog ownership is very often associated with regular physical activity (in terms of regular dog walking) which has important health benefits for adults (Christian et al. 2013). Also, there is evidence that dog owners who walk their dogs tend to have less body weight than non-owners or owners who do not walk (Coleman et al. 2008). However, the majority of adults-owners of dogs have positive intention to walk and this could serve as a target for intervention leading to increase of physical activity (Sterneberg van der Maaten et al. 2016). Concerning childrens' body weight and dog ownership, there are a few studies exhibiting that children-owners are slightly more physically active (Owen et al. 2010). To the contrary, a recent study (Westgarth et al. 2017) supports that current amount of physical activity with dogs is not able to alter childrens' weight or fitness status, after taking into consideration weight, health outcomes and actual engagement with dog walking.

The positive psychophysiological results of human-animal bond are attributed to activation of oxytocin system (Beetz et al. 2012), resulting in decrease of cortisol, epinephrine, and norepinephrine levels in plasma, elevated parasympathetic and diminished sympathetic nervous system activity, lower heart rate and blood pressure. Also, in a recent study in Sweden, dog ownership has been correlated to lower risk of cardiovascular disease in single-person households and lower all-cause mortality in the general population (Mubanga et al. 2017), probably due to reducing psychosocial stress factors.

Also, dogs are used in Animal Assisted Therapy (AAT) which is a goal oriented, planned and structured therapeutic intervention directed by health, education and human service professionals and may enhance treatment outcomes (Jones et al. 2018).

Current therapeutic indications of AAT for medical purposes include psychiatric patients (Barker et al. 2003), pediatric surgery (Calcattera et al. 2015), cancer patients (White et al. 2015), palliative care (Engelman 2013), and posttraumatic stress (Yount et al. 2012). According to preliminary results (as it is a recently adopted method by health professionals), patients may potentially benefit from AAT in terms of positive emotional responses, reduction in stress, pain, fatigue and better communication with health care professionals.

Finally, any dog trained to do work or tasks in order to facilitate an individual with a disability (physical, sensory such as impaired hearing or vision) is defined as a service dog (Muramatsu et al. 2015). Owning a service dog usually brings benefits to the quality of life of persons with disabilities, contributing to their independence, learning and working abilities, community integration and psychological well being (Allen and Blascovich 1996, Hall et al. 2017).

Friedmann's study in 1980 suggested that the reduced mortality rate of coronary disease patients-pet owners occurs due to the effect of human animal interaction on psychological risk factors (Patronek and Glickman 1993). The soothing result of dog-human interaction has been linked to positive impact on physiological factors (Allen et al. 2002). Also, the increased overall physical activity of dog-owners is considered to improve mental health (Ahn and Fedewa 2011). Another recent study (Gadomski et al. 2015) has shown that children who have a pet-dog are less probable to exhibit childhood anxiety, especially social and separation anxiety. Also, contact with dogs can improve mood and produce positive emotions in children and adults with physical or mental health problems. Nevertheless, there is some research studies which state that human-animal interaction is not always beneficial (Straatman et al. 1997, Gillum and Obisesan 2010).

In general, based on the present research evidence and literature, it is widely accepted that contact with the dogs may enhance social skills in children and adults with or without mental problems. It has been demonstrated that pet owners have more friendly interactions with the neighborhood than others (Wood et al. 2005) and social contact is associated with improved mental health (Holt-Lunstad et al. 2010). Moreover, by attending events such as dog shows or walking the dog, owners with common habits and preferences may meet and interact and thereby extend their sense of social connection. These facts indicate that owning a dog may enhance social interaction and connection among persons (Sable 1995).

d) Interaction of dogs with sensitive groups of population

Children

It is well known that children instinctively form emotional attachments with adult human parents or even caregivers for purposes of survival (Hawkins et al. 2017). Later human relationships are affected based on this early experiences while there is evidence that animals-children relationships are characterized by a secure attachment including an affectional bond, special friendship, secure base (Beck et al. 2008). Owning a pet gives the child the opportunity of taking responsibilities for another being, learning new skills, practice and developping a moral and prosocial behavior (Melson 2003). In a recent study, 80% of children asked reported that they felt love for their pets, 83% of those with pets reported that their pet made them happy, 76% reporting that their pet was their best friend, 62% reporting that they would feel lonely without it, and 52% reporting that they felt that their pet could understand if they were sad or upset and it tried to comfort them (Hawkins et al. 2017).

Elderly

It is well documented that life expectancy has been extended and human population is rapidly aging. In almost every country, population growth poses a significant challenge to financial markets, social protection, labour demand. Data from World Population Prospects-2017 Revision-United Nations demonstrates that the number of those over 60 years old is estimated to double by 2050 and reach 3.1 billion persons in 2100. Dog ownership is widespread among older people. The United States National Council for Aging Care suggests that owning a pet is beneficial for elderly people, providing that they make a correct choice of the animal based on their health condition. As it was reported before, even a short contact with pets has physical and psychological benefit for human health. In a very recent study focused on American citizens over 50 years old, a percentage of 51.5% owned a pet while the majority of them owned a dog. They reported positive relationship between them and their pet; over 80% referred to it as their friend and a companion to talk and play while almost 93% of them think that it greatly contributes to their happiness (Mueller et al. 2018). Also, regarding this age group dog ownership may facilitate social interactions. Risks of owning a dog include increased likelihood of falls (Stevens et al. 2014) as well as catching a zoonotic infectious disease. Nevertheless, as psychological factors deteriorate health condition, the main benefit of dog ownership is that permits individuals to be alone without feeling lonely, eliminating stress and other psychological burdens (Steele 2008).

Immunocompromised

Nowadays, a percentage of the population is immunocompromised or with medical conditions requiring immunosuppression (such as cancer, infection with *HIV*, autoimmune diseases) (Robinson and Pugh 2002). Dog ownership offers important benefits to these persons who try to confront their disease. A study conducted in 1999 reported that *HIV* patients who simultaneously owned a dog felt less depression than those who did not have regular contact with pets (Siegel et al. 1999). Providing simple and practical instructions for handling pets to immunocompromised persons by veterinarian and medical professionals will allow them to safely interact with their pets. Risk assessment of dog ownership by immunodeficient patients requires deep knowledge of zoonotic micro-organisms colonizing dogs (Steele 2008).

Acquiring a zoonotic infection is the most prominent risk for these persons due to the insufficient response of their immune system. Bites resulting in cellulitis (Brook 1987) are more frequently characterized by polymicrobial infections with both aerobic and anaerobic bacteria (most frequently: *Pasteurella, Streptococcus, Staphylococcus, Neisseria, Fusobacterium, Porphyromonas, Prevotella*) (Abrahamian and Goldstein 2011, Talan et al. 1999). A careful choice of dog (healthy dog over one-year-old) along with a careful health management (regular visits to the veterinarian, annual vaccination, neutering, yearly examinations for *Salmonella, Campylobacter, Giardia, Cryptosporidium*, keeping the dog indoors, avoiding raw meat as feed) and simple rules of personal hygiene minimize the risks of zoonotic diseases. Consequently, having in mind that the risk for these persons can be kept to minimum, they can be encouraged and take profit from the psychosocial benefits of dog ownership (Steele 2008).

e) Risks and problems of dog ownership

Apart from the benefits deriving from human-dog interaction, sometimes having a dog in the home is challenging. To begin with the most obvious, owning a pet implies specific obligations, some of which are defined by the law. Microchipping, registration and confinement of dogs to your property, neutering, annual vaccinations against rabies virus and regular visits to the veterinarian are some of these, according to laws no 4039/A’15/02-02-2012 and no 4235/B’32/11-02-2014 (in Greece). Consequently, dog ownership goes with increased financial cost along with time and energy required for feeding, walking and playing with it. Moreover, health hazards include bites, scratches, allergies.

However, the most important danger deriving from dog ownership is still represented by zoonoses, as the human-dog bond has grown stronger during the last decades (Alho et al. 2018). Pets have been found to be able to transmit over 60 zoonotic pathogens (Macpherson 2005). According to Centers for Control of Disease and Prevention (CDC) zoonotic agents can be transmitted in every possible way:

-Through direct contact (with the saliva, blood, urine, mucous, faeces, or other body fluids of an infected animal or following a bite or a scratch),

-Through indirect contact (with areas, objects or surfaces contaminated with the zoonotic pathogen),

-Vector-borne transmission (the pathogen is transferred by an insect, i.e. a mosquito, tick, flea),

-Foodborne (ingestion of contaminated food or drink).

People belonging to sensitive groups of5 the population are in increased risk of acquiring a zoonotic disease; children younger than five years old, elderly over 65 years old, immunodeficient persons. The major zoonotic infectious agents found in dogs are presented in the following **Table 1**.

Table 1. The major zoonotic infectious agents found in dogs.

<table>
<tr><td rowspan="11">Parasitic Zoonotic Pathogens</td><td rowspan="3">Protozoa</td><td>Leishmania species (spp.)</td></tr>
<tr><td>Giardia duodenalis</td></tr>
<tr><td>Cryptosporidium spp.</td></tr>
<tr><td rowspan="2">Cestodes</td><td>Echinococcus spp.</td></tr>
<tr><td>Dipylidium caninum</td></tr>
<tr><td rowspan="3">Vector-borne helminths</td><td>Dirofilaria repens</td></tr>
<tr><td>Thelazia callipaeda</td></tr>
<tr><td>Onchocerca lupi</td></tr>
<tr><td rowspan="3">Nematodes</td><td>Toxocara canis</td></tr>
<tr><td>Ancylostoma</td></tr>
<tr><td>Uncinaria stenocephala</td></tr>
<tr><td rowspan="6">Bacterial Zoonotic Pathogens</td><td colspan="2">Campylobacter spp.</td></tr>
<tr><td colspan="2">Salmonella spp.</td></tr>
<tr><td colspan="2">Brucella canis</td></tr>
<tr><td colspan="2">Yersinia enterocolitica</td></tr>
<tr><td colspan="2">Pasteurella spp.</td></tr>
<tr><td colspan="2">Capnocytophaga canimorsus</td></tr>
</table>

	Bordetella bronchiseptica
	Coxiella burnetii
	Leptospira interrogans
	Staphylococcus intermedius
	Methicillin resistant Staphylococcus aureus (MRSA)
Viral Zoonotic Pathogens	Rabies
	Rotaviruses
	Noroviruses
	Coronaviruses

f) Dogs' interactions with wildlife

Although dog is considered as human's best companion, it is also a serious threat for wildlife species (Doherty et al. 2016). As they range from feral (free-roaming) to owned (completely restricted), they interact with wildlife in many ways. It was demonstrated that dogs represent the third introduced mammalian predator (after cats and rats) and is considered as a potential or known threat for over 150 species (Doherty et al. 2017). Their negative interaction with wildlife species includes:

a) Direct predation (Ritchie et al. 2014),

b) Fear-mediated behavioural changes (Banks and Bryant 2007, Zapata-Ríos and Branch 2016),

c) Competition (Vanak et al. 2014),

d) Harassment (Weston and Stankowich 2014),

e) Hybridisation (Bassi et al. 2017), and

f) Disease transmission (Furtado et al. 2016).

Accurate evaluation of the negative impact provoked by dogs to wildlife species is hard to be exhibited, due to the small number of existing studies quantifying this impact (Hughes and Macdonald, 2013). Nevertheless, there are some examples of this events:

- *Canine Distemp*er epidemic which lead to 30% decrease of the lion population in Serengeti (1994) linked to unvaccinated dog populations in the area (Roelke-Parker et al. 1996, Viana et al. 2015).
- Rabies epidemic which threatened Ethiopian wolf conservation (Sillero-Zubiri et al. 1996, Lembo et al. 2005)

In any case, the impacts and interactions among domestic dogs and wildlife species are yet to be further investigated and understood. Management schemes and actions should be designed and implemented, based on the different sociological, ecological and climatic conditions of each area or country. Special attention should be given to the probability of spread of zoonoses in rural interfaces where humans, dogs, livestock and wild animals live in proximity (Daszak et al. 2000, Kalerna-Zukosoka 2003).

1.2 European Brown Hare

The European Brown Hare (*Lepus europaeus*) is a species of hares which lives in Europe and parts of Asia. It belongs to the order *Lagomorpha*, Family *Leporidae*, Genus *Lepus* along with 31 species. They are herbivorous and their predators include birds of prey, canids and felids. They are characterized by high-speed endurance which facilitates them to escape from their predators. It is classified as a species of least concern by the International Union for Conservation of Nature because it can be found within a wide range and in abundance. Brown hares have been traditionally hunted for centuries in Europe and they represent one of the most important game species.

Lepus europaeus is consisted of 15 subspecies: *Lepus europaeus caspicus*, *L. e. connori*, *L. e. creticus*, *L. e. cyprius*, *L. e. cyrensis*, *L. e. europaeus, L. e. hybridus*, *L. e. judeae*, *L. e. karpathorum, L. e. medius*, *L. e. occidentalis*, *L. e. parnassius*, *L. e. ponticus*, *L. e. rhodius*, *L. e. syriacus*, and *L. e. transsylvanicus* (Hoffmann and Smith 2005).

a) Geographical range and habitat

Lepus europaeus can be found as a native species in the majority of continental European countries: Austria, Germany, Hungary, Czech Republic, Serbia, Slovaka, Slovenia, Albania, Bosnia and Herzegovina, Croatia, Bulgaria, Romania, Spain, Switzerland, Belgium, Estonia, Latvia, Lithuania, Belarus, Poland, Netherlands, Italy, Russian Federation, Ukraine Moldova, Montenegro and also in Greece. Also, it can be found as a native species in Turkey, Syrian Arab Republic, Iran, Islamic Republic of Iraq and it has been introduced in United Kingdom, Ireland, Sweden, United States, Canada, Brazil, Chile, Argentina, Uruguay, Australia (Tazmania), New Zealand (Hacklander and Schai-Braun 2019).

European brown hares tend to live to open fields with hedges, ditches, brushes which serve as coverage and offer food sources. Their population density is associated with low altitudes (below 200 meters), 40 to 60 days of snow coverage, 50 to 700 millimetres of annual precipitation, and a mean annual air temperature of around 10 °C (Pikula et al. 2004). They mainly prefer dry distinct and mild winters and they thrive in mixed and open farmlands.

b) Behaviour and ecology

Hares are primarily nocturnal and spend a great proportion of their time foraging (Chapman and Flux 1990). During daytime, European hares remain partly hidden in a depression in the ground called "form" which they scrape. It is very difficult to define their exact numbers, as they are very well concealed in these "forms" and it is hard to detect them. In this way, hares protect themselves and they move only when detected to evade their predators. They can develop speed up to 75 kilometers (km) per hour and they rely on their speed to outrun them. As feed, they mainly prefer wild grasses and weeds but they can also feed on crops (90% of soft greens, 5.5% woody plants, 2.2% root crops, 1.7% grain crops and 0.5% forest plants).

Lepus europaeus is a sedentary species as it lives and moves within a specific geographical area, which is known as home range. Home range of hares is a relatively small area. The animals may move up to 1.8 km in order to feed on graze. They are mostly quiet and solitary but group feeding is common as an extra protection measure against predators. Their breeding season extends from January to August but their reproductive behaviour escalates in spring, leading to the well-known phrase of "Mad March Hares" which is used in English language to describe a human as mad and unpredictable as a hare in March.

c) Infectious Diseases affecting hares

European Brown Hares are considered as an important marker of environmental changes and infectious diseases (including vector-borne diseases) circulating in a specific area due to their short lifespan, their interactions with the environment and their frequent contact with ticks, mosquitos and sandflies. Many of these pathogens have a serious impact on hares' mortality rates. Apart from that, hares can intersperse various pathogens, some of which may be zoonotic (*Borrelia* spp., *Listeria* spp., *Toxoplasma gondii*, *Leptospira* spp., *Fra*ncisella tularensis*) (Treml et al. 2003, Bartling et al. 2004).

-Viral agents: *European Brown Hare Syndrome Virus* (*EBHSV*)*, Rabbit Haemmorhagic Disease Virus 2* (*RHDV2*)*, Myxoma Virus* (*MYXV*)*, Hare Fibroma Virus, Crimean Congo H*aemorrhagic Fever Virus* (*CCHFV*)*, Hepatitis E Virus* (*HEV*)*, Pestiviruses*.

-Bacterial agents: *Yersinia* spp., *Brucella* spp., *Francisella tularensis, Maenhaemia Haemolytica*.

-Parasitic and Protozoal agents: *Toxoplasma gondii, Leishmania* spp., coccidia, nematodes.

d) Threats

European brown hares are concerned as a species a species of least concern by the International Union for Conservation of Nature. Nevertheless, their populations have been reduced across Europe during last decades. The main reasons for this decline are the intensification and changes in agricultural practices (reductions of grass areas, augmentation of fields, fragmentation of natural habitats), predation and infectious diseases (for instance European Brown Hare Syndrome).

Concerning predation, European Brown hares along with the rest lagomorphs are mainly prey for many predator mammals but also large predator-birds. Red fox (*Vulpes vulpes*) is their most important predator, as mortality of 50% of adult hares is caused by foxes and they constitute one of the most significant part of their diet, representing approximately 50% of the biomass which they consume, especially during spring (Goszczyński and Wasilewski 1992, Bakaloudis et al. 2015). Other animal species included in their predators are: weasels, cats, bobcats, lynx, civets, coyotes, golden eagles (Kurta 1995, Watson and Brockie 1997).

1.3 *Canine Parvovirus*

a) Taxonomy and Structure

Parvoviridae are a family of small, rugged, DNA viruses. There are currently over 75 species, including 13 genera and two subfamilies. These two subfamilies *Parvovirinae* and *Densovirinae* infect vertebrates and insects respectively. *Parvovirinae* includes five genera *Parvovirus, Erythrovirus, Dependovirus, Amdovirus* and *Bocavirus*. *Canine parvovirus* (*CPV*) belongs to genus *Parvovirus* and has been included in the unique species Feline panleukopenia virus together with *feline panleukopenia virus* (*FPV*), *mink enteritis virus* (*MEV*) and *raccoon parvovirus* (*RPV*) (Siegl et al. 1985, Cotmore and Tattersal 1987).

In the 1970s, *CPV* emerged from feline leucopenia virus; between them there is more than 98% sequence homology. Six coding nucleotides in the *VP2* gene differ from *FPV* genome and result in *VP2* residue 93 Asparagine instead of Lysine and *VP2* residue Asparagine instead of Aspartate (Chang et al. 1992, Truyen et al. 1995). *CPV2 is* genetically distinct and unrelated to *Canine minute virus* (former *Canine Parvovirus type 1*) which now belongs to the genus *Bocavirus* (Pratelli and Moschidou 2012; Nandi and Kumar 2010).

Parvoviruses are small (diameter of 25 nm), non-enveloped viruses, as it is demonstrated by their name; parvus means small in Latin. The virion consists of a capsid which encloses a linear single-strand DNA molecule of approximately 5200 nucleotides (Muzyczka and Berns 2001). Parvoviral genome consists of two Open Reading Frames (ORFs) and certain genes encoding for structural (*VP1*, *VP2*) and non-structural (*NS1*, *NS2*) proteins. The role of the capsid is protection of the viral genome during transfers to other cells and various functions necessary for its delivery in the nucleus and infection (Harbison et al. 2008). The capsid is an icosahedron formed by 60 copies of combinations of the viral proteins *VP1* and *VP2* and *VP3*. *VP2* is the most abundant structural protein (90% of total viral capsid) while *VP3* is created following the proteolytic cleavage of approximately 19 residues from the N edge of a part of the *VP2* molecules (Weichert et al. 1998). *VP1* includes 143 N-terminal amino acids more than *VP2*. The structure of the surface of the capsid is complicated, including pores at the exterior at the 5-fold axes, spikes around 3-fold axes, concave regions at the 2-fold and 5-fold axes of symmetry (Tsao et al. 1991). The viral capsid of *CPV* binds to transferrin receptor type 1 (TfR) of the cell membrane and enters in carnivore host cells resulting in infection, while it serves as target for the antibodies which are subsequently produced (Hueffer et al. 2003).

b) Evolution

Parvoviruses of domestic cats (*Felis catus*) and other carnivore species, [such as American mink (*Neovison vison*) and raccoon (*Procyon lotor*)] have been known for many years and have been generally described as *Feline Panleukopenia Virus* (*FPV*) or related variants. Nevertheless, the variant that infects dogs (*CPV*) emerged in the 1970s with outbreaks in U.S.A., Australia and then many other countries, (probably through adaptation of an *FPV*-like *parvovirus* of wild carnivores) due to mutations that changed surface residues in two regions of the capsid (Chang et al. 1992). There are at least six or seven amino acid (aa) changes between *FPV* and *CPV-2*, more frequently situated in the *VP2* domain interacting with the host-cell transferrin receptor (TfR) which may resulted in preference for canine receptor TfR (Shackelton et al. 2005). Apart from that, *CPV* (unlike *FPV*) evolves very quickly, demonstrating substitution rates comparable to those of RNA viruses (Battilani et al. 2006, Decaro et al. 2008, Decaro et al.2009).

As time passed, exposure to the virus and vaccinations contributed to acquisition of immunity in canine populations (lower mortality rates, restriction of further spreading). However, host-immunity pressure along with other factors has led to genetic mutations and the emergence of new genetic and antigenic variants. Later, two distinct antigenic variants emerged (*CPV-2a* in 1980s, *CPV2b* in 1991, *CPV-2c* in 2001), which replaced the original *CPV-2* and spread in the canine population worldwide. They differ from the initial *CPV-2* strain in five or six amino acid residues of the *VP2* protein while *CPV-2a* differs from *CPV-2b* in one or two residues (Decaro and Buonavoglia 2012). Recent *CPV-2a* variants may differ from *CPV-2b/2c* strains only in one residue found at 426 of *VP2* protein (Decaro and Buonavoglia 2012). Mutations continue to accumulate throughout the years, more often affecting the *VP2* region between residues 267 and 498, which presents great variability among *parvoviruses*, as it is found on the capsid. However, implications of the majority of these changes usually remain unknown. Consequently, the nomenclature of all these strains has been and is still confusing (Zhou et al. 2017).

c) Pathogenesis

Infection with *Canine Parvoviruses* occurs following oronasal exposure to contaminated faeces in the environment. Vectors carrying the virus include people, instruments (i.e. veterinarians and veterinary medical equipment), insects, rodents, and dogs’ hair coat. Incubation period of *CPV-2* has been described to be 7-14 days in the field, but incubation period of strains *CPV-2a, CPV-2b* is shorter (approximately 3-7 days). Dogs of any age, breed or sex may be infected with *Canine Parvovirus* but puppies of age 6 weeks to 6 months are at bigger risk. Also, Rottweilers, Labrador retrievers, American Staffordshire terriers, German shepherds face an increased risk of acquiring a parvoviral enteritis.

Target tissues of *CPV* are lymphoid tissues of the oropharynx, mesenteric lymph nodes, and thymus where virus replication begins and then it is transferred to the intestinal crypts following marrow. Nevertheless, it can be found in other organs as well (i.e. myocardium, lungs, spleen, liver, kidney, brain) (Elia et al. 2007).

As it is known, the small intestine is consisted of layers: serosa, muscularis, submucosa, and mucosa. It is very important for digestion, secretion and absorption and it includes folds (plicae circulares), villi (systematically distributed mucosal projections) and microvilli, adjacent to crypts of Lieberkuhn (glands). The epithelium of the villi is made up of columnar absorptive cells called enterocytes, their apical surface (microvilli) and goblet cells. Enterocytes and villi contribute to increasing the surface available for absorption while goblet cells mainly secrete mucus. The epithelium of villi stretches down into the lamina propria forming the crypts. The later contain Paneth cells of the innate immune system (secretion of antimicrobial peptids), endocrine cells, lymphocytes, enterocytes, goblet cells and the mitotically active population of stem cells. These stem cells divide and then move towards the lumen and differentiate into enterocytes or goblet cells (every 1-3 days).

Parvovirus infects the enterocytes populating the intestinal crypts resulting in mucosal destruction and collapse affecting regular replenishment of cells and the length of villi, while causing mucosal and serosal haemorrhage, maldigestion and malabsorption diarrhea. It also infects the precursors of leukocytes and lymphoid cells leading to neutropenia and lymphopenia (usual laboratory findings in parvoenteritis). Secondary bacterial infection may deteriorate the situation by induction of bacteraemia, endotoxaemia and disseminated intravascular coagulation. Shedding of *CPV* is observed on the third or fourth day and can be detected in faeces for seven to ten days post infection. Local immunological response is developed which blocks *CPV* shedding while detection of antibodies begins from third or fourth days post infection and may last to one year.

Viraemia may last from 1 to 5 days (Pollock and Carmichael 1982). Then, the virus is mainly located to the gastrointestinal tract epithelium (tongue, oral and esophageal mucosae, and small intestine), the lymphoid organs (thymus, lymph nodes) and bone marrow.

d) Clinical and laboratory findings

Infection with *canine parvovirus* may be presented with two forms: a) intestinal form which is much more common and b) cardiac form. Intestinal form is characterized by vomiting (usually initial sign) severe haemorrhagic diarrhea (faeces remain fluid until recovery or death), dehydration, lethargy, weight loss, anorexia, fever. The symptoms are usually presented after an incubation period of 3-7 days. The severity of the disease depends on the maternally derived antibodies (MDA) titers at the moment of infection (Decaro and Buonavoglia 2012). Specifically, puppies of an immunized mother are protected from *CPV* infection for the first weeks of their life provided that they have consumed sufficient quantity of colostrums. However, as MDA titer is reduced, susceptibility to *CPV* infection increases. Other factors associated with parvoenteritis include overcrowding, stress, malnutrition, intestinal parasitism, other enteric infections, winter or spring time, free-roaming, veterinary health care (Curi et al. 2016, Kilian et al. 2018). Cardiac form (infection of myocardium) could occur following the infection of myocytes with the virus in the 1-3 weeks after birth of seronegative puppies, leading to myocardial necrosis, pulmonary edema and acute heart failure or delayed progressive cardiac failure. Nevertheless, as the majority of dams is vaccinated, this form of the disease is rarely encountered.

Compatible laboratory findings include leukopenia, lymphopenia, thrombocytopenia or thrombocytosis, anaemia, pancytopenia, neutrophilic leukocytosis and monocytosis (Goddard et al. 2008, Castro et al. 2013). In particular, leukopenia (neutropenia and/or lymphopenia) is a very frequent sign of parvoenteritis observed in the complete blood count. Leukopenia (white blood cell counts below 2000–3000 cells/mL of blood) is caused by the infection and destruction of hematopoietic progenitor cells in bone marrow and lymphoid which is further exacerbated by the extended inflammation of the intestinal tract (Mylonakis et al. 2016). Other non-specific serum biochemistry changes may be hypoproteinemia, hypoalbuminemia, hypoglycemia, hypoglobulinemia, as well as electrolytic abnormalities hypocalcemia, hypokalemia, hyponatremia, hypochloremia and hypomagnesemia (Kalli et al. 2010). Non-invasive diagnostic markers such as acute phase proteins (C-reactive protein, haptoglobin, ceruloplasmin) are sometimes used for defining disease prognosis during research projects but they are not thoroughly evaluated yet (Kocaturk et al. 2010).

e) Pathological findings

Haemorrhagic enteritis of the small intestine (thickened and discolored intestinal wall and watery, mucoid or haemmoragic content), fluid in the abdominal cavity enlargement and congestion of thoracic, abdominal and Peyer’s patches are the characteristic gross necropsy lesions of dogs dead by parvoenteritis. Histopathologically, multifocal necrosis of the crypt epithelium, loss of crypt architecture, villous atrophy and/or necrosis, necrosis of Peyer's patches and mesenteric lymph nodes, and bone marrow hypoplasia are observed (Atalay Vural and Alcigir 2011).

f) Diagnosis of canine parvoenteritis

Clinical diagnosis of canine parvoenteritis may be confusing and indecisive as the symptoms are included in the differential diagnosis of many other viral agents (i.e. *coronaviruses, morbilliviruses, rotaviruses, noroviruses, adenoviruses*) (Greene and Decaro 2012). Consequently, it is recommended that the diagnosis should be confirmed by laboratory testing. Faecal specimens (collected with appropriate swabs) are the biological material more frequently used for laboratory confirmation of *CPV* infection. If the animal is dead, intestinal content could be used for post-mortem laboratory analysis. Also, blood samples can be used due to the long lasting *CPV* viraemia but in the later stages of infection (Decaro et al. 2005). Identification of the causative agent of the diarrhea is very important for establishing a prognosis for a puppy as well as for preventing an outbreak in cases of kennels or hospitals.

g) Traditional methods

Currently, immunochromatographic-strip based rapid tests detecting *CPV* antigen are the most widely used diagnostic tool for detecting *CPV*, especially by veterinary practitioners (Desario et al. 20005). These commercial tests are usually based on the sandwich technique, using one or two monoclonal antibodies (Schmitz et al. 2009). Despite of some concerns about the detection of new strains *CPV 2c*, a recent study demonstrated that the detection rate of this strain is similar to that of *CPV 2a* and *2b*. Poor sensitivity of these assays could be attributed to lower amounts of virus in the faeces during later stages of infection, using the same mAb for detection and capture, presence of *CPV* antibodies in the lumen of the intestine (Sharma et al. 2018). However, these kits remain very popular among veterinary clinicians due to their advantages (low cost, immediate results, easy-to-perform). Due to lower sensitivity of these tests, in cases of questionable results or animals living with many others, it is recommended that the results should be confirmed by other laboratory methods.

Haemagglutination (HA) and haemagglutination inhibition (HI) are alternative traditional techniques for *CPV* detection, which were widely used in the 1980s (Carmichael et al. 1980). They are simple, inexpensive and provide results within the day (4 hours). However, they are carried out only in equipped laboratories and fresh erythrocytes are required (usually porcine). Many factors could alternate the results (i.e. incubation times, red blood cell concentration and quality, *CPV* strains without haemagglutination capability, subjectivity of test operator) (Desario et al. 2005). Also, virus isolation on cell cultures can be used (only in specialized laboratories with trained personnel and cell culture capability) but it is time consuming (5-10 days). However, viral isolation of *CPV* is challenging, as it frequently causes slight cytopathic effect on cell cultures, difficult to be seen and it depends on the stage of the infection (Mochizuki et al. 2008). In general, the main drawback of these methods is their low sensitivity due to binding of the *CPV* virions with antibodies in the lumen of the gut (Desario et al. 2005). Apart from the above methods, electron microscopy and immunohistochemistry may be available as diagnostic tests in specialized laboratories, but they are rarely used.

h) Molecular methods

In general, molecular methods are more sensitive than traditional techniques and they are not affected by the host immune response. Initially, a nucleid acid detection assay had been designed by Remond et al. in 1992 and was suggested as an alternative diagnostic method of *CPV*. Later, several conventional non-quantative PCR methods of increased specificity and sensitivity PCR assays were developed (Mochizuki et al. 1993b, Pereira et al. 2000). Apart from conventional PCR methods, a real-time PCR method was suggested which simultaneously permits the detection and quantification of DNA in the faeces of diarrhoeic dogs (Decaro et al. 2005) while some time later a loop-mediated isothermal amplification assay was proposed as an alternative PCR based method.

Molecular methods are also used for virus discrimination between vaccine and wild strains of the virus, whereas this is not often required by veterinary practitioners, but it is used for epidemiologic purposes during studies. However, the results of genotyping are not always definitive and more than one method may be required in order to achieve high certainty of which the strain is. At first, Pereira et al. suggested a genotyping PCR method in 2000 which cannot longer discriminate novel *2a* strains from *2b* strains or even novel *CPV* type *2c* strains (Buonavoglia et al. 2001). Sequence analysis can also be applied towards this end by using conventional PCR with the primer pair 555for/555rev or Real-Time PCR based on MGB probe technology which detects SNPs at codon 426 of *VP2* protein (Decaro et al. 2005, 2006).

i) Discrimination between vaccine and field strains

Vaccination of puppies and adult dogs may provoke confusion in the diagnostic tests of *CPV* in faeces of animals with diarrhea. This happens due to the replication of the modified live virus of the vaccines and its shedding in faeces for few days after vaccination (Decaro et al. 2006). In this way, false positive results may occur, if immunochromatography, haemagglutination or PCR are used, leading to disregard other enteropathogens responsible for the symptoms. In general, *CPV* vaccines are considered to be very safe and it is unlikely that they could induce gastroenteritis. Characterization of *CPV* strains is difficult and indecisive with the traditional methods, especially when coexistence of vaccine and wild strains occurs. Towards this end, a PCR protocol was presented in 1995 by Senda et al., but it was not always able to discriminate the old (vaccine) from the wild strains (Decaro and Buonavoglia 2012). Later another technique was suggested by Decaro in order to overcome the previous limitations. In conclusion, nowadays discrimination between vaccine and wild strains could be done, especially if there is need to confirm that post vaccination diarrhea occurs due to vaccine or wild strains. This is not usual and recent studies have shown that usually it is displayed because of wild strains incubating at the time of vaccination (Decaro et al. 2007). Nevertheless, it is well established that the greater part of these infections in puppies (which are the main population under vaccination protocol) are presented at the time when maternal derived antibodies (MDA) decline.

j) Prophylaxis

Prevention against *CPV* gastroenteritis relies mainly on vaccination programmes. Vaccination is not obligatory by law but the majority of dog-owners are persuaded by veterinarians to implement it to their pets. In general, modified live virus (MLV) vaccines are used under several commercial labels and they usually contain the original type *CPV-2* or *CPV-2b*. Sometimes vaccinations fail due to interfering levels of maternally derived antibodies (MDA) and this is the reason why it is crucial that the right time for vaccination should be selected (after waning of MDA). According to the Vaccination Guidelines Group of the World Small Animal Veterinary Association, an effective way to overcome even longlasting MDA in puppies is to delay the finish of primary vaccination course to 16 weeks of age.

Another issue has aroused since the identification of the novel variant *CPV-2c* regarding the efficacy of vaccines against it. Some researchers even highlighted the need for new vaccines to be manufactured (Blanco and Catala-Lopez 2015). Recent studies on the matter suggested that the commercial vaccines contribute to protection from all *CPV* strains infection, including *CPV-2c* (Wilson et al. 2014). Nevertheless, limitations observed in these studies (i.e. limited number of small sample sizes, short follow-up) indicate that there is need for further research on this issue (Blanco and Catala-Lopez 2015).

1.4 *Salmonella* spp.

a) Characteristics of *Salmonella* spp.

Salmonella spp. is a genus of the family *Enterobacteriaceae* and they are rod-shaped (bacilli) Gram-negative bacteria, non spore-forming with cell diameters 0.7-1.5 x 2.5 μm and peritrichous flagella. This means that they are motile with the exception of serovars adapted to birds, such as *S. Gallinarum* and *S. Pullorum* (Jajere et al. 2019). They are facultative aerobes with optimal growth temperature at 37^{o}C, which means that they can produce adenosine triphosphate (ATP) with oxygen when it is available; otherwise they use electron acceptors or fermentation (Fabrega and Vila 2013). They are primary intracellular pathogens leading to different clinical symptoms in humans and animals. *Salmonella* spp. is catalase positive, oxidase negative and they ferment glucose, mannitol, maltose, mannose and sorbotol to produce acid or acid and gas. Also, they ferment sucrose but rarely adonitol. They do not hydrolyze urea or deaminate phenylalanine but they usually form H_2S, lysine and ornithine decarboxylases. They are negative to Voges-Proskauer but positive to methyl red tests. They are usually urease and indole negative. Identification of the microorganism can be done based on the above biochemical traits. A basic trait of *Salmonellae* is that less than 1% of them are reported to ferment lactose (Ewing 1996). Consequently, lactose fermentation is used as a basic biochemical test for the identifications of *Salmonellae* and the majority of differenting plating media contain lactose (McDonough et al. 2000.

Table 2. Differential biochemical traits of *Salmonella* species and subspecies. (Le Minor et al. 1982).

Species	***S. enterica***						***S. bongori***
Subspecies	***enterica***	***salamae***	***arizonae***	***diarizonae***	***houtanae***	***indica***	
Characters							
Dulcitol	+	+	-	-	-	d	+
ONPG	-	-	+	+	-	d	+
Malonate	-	+	+	+	-	-	-
Gelatinase	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	+	-	+
Growth with KCN	-	-	-	-	+	-	+
L(+) – tartrate[(a)]	+	-	-	-	-	-	-
Galacturonate	-	+	-	+	+	+	+
γ-glutamyltransferase	+*	+	-	+	+	+	+
β-glucuronidase	d	d	-	+	-	d	-
mucate	+	+	+	- (70%)	-	+	+
salicine	-	-	-	-	+	-	-
lactose	-	-	- (75%)	+ (75%)	-	d	-
Lysed by phage O1	+	+	-	+	-	+	d
[(a)]= *d*-tartrate							
* Typhimurium d, Dublin -							
d = different reaction by different serovars							

Serotyping of *Salmonellae* can be done using a scheme suggested by Kauffman and White, which is based on three major antigenic determinants: somatic (O), capsular (K) and flagellar (H) (Brenner et al. 2000). Discrimination is based on their ability to interact with relevant anti-sera. The heat-stable O antigen is the oligosaccharide component of lipopolysaccharide located at the outer bacterial membrane, which serves as the endotoxin of bacterial cell and is released only if the cell is destroyed. Each *Salmonella* serovar may express more than one O antigen (Hu and Kopecko 2003). H antigens are susceptible to heat, alcohol and acids, they are situated on flagella and contribute to the activation of host immune responses. The majority of *Salmonella* spp. have two distinct genes encoding for the flagellar proteins and they express only one each time. These bacteria are called diphasic (phase I and phase II). Each serotype expresses specific phase I H antigens. On the other hand, phase II antigens are non-specific and characterize many serotypes (McQuinston et al. 2008). Finally, the capsular K antigens are heat-sensitive polysaccharides which are found more rarely in *Salmonella* spp. strains. Virulence (Vi) antigens are a subtype of K antigen which cover the O antigen and is produced by strains of serotypes *S*. Typhi, *S*. Paratyphi and *S*. Dublin and they are considered to protect the bacterial cell from complement action when in extracellular environment (Daniels et al. 1989).

b) Nomenclature

Until now, more than 2500 serovars of *Salmonella* have been identified but less than 100 serotypes are linked with human disease, according to CDC (Healy and Bruce 2019). *Salmonella* spp. nomenclature is complex and different systems are used. The World Health Organization Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute is responsible for the definition and maintenance of *Salmonella* serotypes. Nomenclature is based on the serologic definition of O (somatic) and H (flagellar).

Salmonella taxonomy changed in 1973, as it was proved that all serotypes and subgenera I, II and IV along with Arizona serotypes were genetically related by DNA-DNA hybridization (Crosa et al. 1973). This means that they all belong to the same single species except for *Salmonella bongori*, previously known as subspecies V (Reeves et al. 1989). Later, in 1986 the Subcommittee of *Enterobacteriaceae* of the International Committee on Systematic Bacteriology at the XIV International Congress of Microbiology suggested that the type species for *Salmonella* should be changed to

Salmonella enterica instead of *Salmonella cholerasuis*, because there is no serovar sharing this name (Penner 1988).

In 2002, the Judicial Commission of the International Committee for Systematics of Prokaryotes in the Judicial Opinion 80 approved the request for the change in the nomenclature system for *Salmonella* and from January 2005, "*Salmonella choleraesuis*" would be modified to "*Salmonella enterica*" with "*Salmonella enterica*" becoming the type species of the genus *Salmonella* (Ryan et al. 2017). This particular decision complies with the bacteriological code; however, it fails in putting an end to the use of *S*. choleraesuis as the type strain (Heyndrickx et al. 2005).

The Judicial Commission ruled that the *Salmonella* genus is comprised of two species, called "*Salmonella enterica*" and "*Salmonella bongori*." "*Salmonella enterica*" is divided in six subspecies biochemically and genetically differentiated, which are referred as follows:

I, *S. enteric* subsp.*enterica*;

II, *S. enterica* subsp.*salamae*;

IIIa, *S. enterica* subsp.*arizonae*;

IIIb, *S. enterica* subsp.*diarizonae*;

IV, *S. enterica* subsp.*houtenae*;

VI, *S. enterica* subsp.*indica*.

c) Current Nomenclature

The nomenclature system proposed by Tindall et al. is the system that is currently used by the World Health Organization and the American Society of Microbiology

(Tindall et al. 2005). The term “serovar” may refer to the antigenic formula of various subspecies or the serovar name which is used in practice for isolates of *S*. enterica subsp. enterica (subspecies I). For serovars of *Salmonella enteric*a subsp. *enterica*, the names were given depending on the disease caused by the infection, the geographic area of their origin or their typical habitats. In the other *Salmonella enterica* subspecies and in *Salmonella bongori* serovars, antigenic formulae are used according to Kauffmann-White-Le Minor scheme (Grimont and Weil 2007).

For the first mention in a text, *Salmonella enterica* is used and then the subspecies is named (*Salmonella enteric*a subsp. e*nterica*). This is then followed by “serovar” or the abbreviated version “ser.” and the name of the serovar (Guibourdenche et al. 2010). The name is written in nonitalicised Roman alphabet letters with the first letter capitalized. Other mentions of the name can be condensed with “*Salmonella*” being followed by just the serovar name. A full serovar name is only reffered to isolates of S*almonella enterica* subsp. *enterica* which meet the full and exact antigenic definition. In each other case, the antigenic formula becomes the serovar name.

For the other five *Salmonella enterica* subspecies, serovars are mentioned according to antigenic formulae, the subspecies name is given in Roman letters (not italicised), and antigenic formulae are then listed as follows: O (somatic) antigens: Vi (when present): H (flagellar) antigens (phase 1): H antigens (phase 2, if present) (Nataro et al. 2011). For serovars of S. bongori (which was formerly classed as subgenus V), V is still used.

d) Host specificity

The natural reservoir of *Salmonellae* is the intestines of warm-blooded and cold-blooded animals, which frequently serve as subclinical carriers shedding the pathogen. Moreover, they can be found everywhere and they survive up to 9 months or more in wet, muddy locations infected with animal faeces or feed (Quinn et al. 2006). For these reasons they are considered as "universal pathogens" (Falkow and Mekalanos 1990).

It is interesting that they exhibit different pathogenicity depending on the species of animals. This happens due to their different level of ability to adapt to hosts. The adaptation of serovars to a specific animal species leads to increased virulence against this species along with reduced virulence against other animal species. This phenomenon is defined as "host specificity" (Bäumler and Fang 2013).

Moreover, infection with *Salmonellae* does not always result in identical clinical symptoms. Several factors affect the manifestation of clinical symptoms, such as: serovar, number of bacteria ingested, animal species, age of the animal, state of health of the host (Wallis and Barrow 2005).

Consequently, serovar-host specificity of S*almonellae* divides them in three groups of serovars depending on their host range:

a) Serovars with broad host range (known as unrestricted or ubiquitous serovars) which infect all animals (such as *S*. Typhimurium and *S*. Enteritidis), usually inducing self-limited gastroenteritis.

b) Serovars which cause severe infection in specific hosts which they prefer and are excreted without symptoms or provoke mild symptoms when infect different hosts –

usually phylogenetically relative organisms (known as host-adapted or host-specific serovars, such as *S*. Dublin, *S*. Cholerasuis) (Uzzau et al. 2000).

c) Serovars which are strictly restricted to one specific host (known as host-restricted serovars), causing severe systemic infection, sometimes even fatal, by exhibiting high tropism for lymphatic organs of its host (i.e. *S*. Typhi, *S*. Gallinarum) (Wallis and Barrow 2005).

Adaptation to hosts is a complicated procedure and is determined by numerous genes and their products. The pathogenicity of *Salmonella* strains in a host is affected by the selective pressure within the particular host and its environment (Thiennimitr et al. 2012). Common methods used by *Salmonella* for successful adaptation and infection of the host include acquisition of novel virulence determinants and plasmids (horizontal gene transfer), gene deletion, allele variations, gene mutations, reduced metabolic flexibility. Apart from these genetic factors, the health state of host, the availability of amino acids and the ability of each strain to replicate in the host play important role for the virulence (Tierrez and García-del Portillo 2005)

e) Evolution

The evolution of pathogenic *Salmonella* from a non-pathogenic ancestor, probably common with that of *E. coli* (120 million years ago), arose due to the acquisition of key virulence genes via horizontal gene transfer (Groisman and Ochman 1996). Virulence genes are located on pathogenicity islands (large cassettes composed of a series of genes and operons resulting in a specific virulent phenotype) which include approximately 10-200 kb of genomic DNA (Marcus et al. 2000). These islands exist in pathogenic bacterial strains while they are absent from the genomes of nonpathogenic members of the same or related species and are very important for the pathogenesis of *Salmonella enterica* infections (Groisman and Ochman 1996).These islands are known as *Salmonella* pathogenicity islands (SPI) and play a crucial role in host cell invasion and intracellular mechanisms of pathogenesis (Hensel 2004). Key virulence genes include two types II secretion systems (T3SS-1 and T3SS-2) encoded on SPI-1 and SPI-2. These systems are supramolecular structures in shape similar to a syringe and facilitate invasion of host epithelium and survival in the intracellular environment (Srikanth et al. 2011). Until now, at least, 17 different SPIs have been discovered, and they have been found to encode the major virulence phenotypes, i.e. host-cell invasion and intracellular pathogenesis (Chiu et al. 2005, Vernikos and Parkhill 2006), while five of them are of critical importance.

It is known that the evolution of *Salmonella* spp. occurred in three phases: Initially, the bacteria acquired SPI-1 via horizontal gene transfer with plasmid or phage, which is present in every serotype, while is absent from genome of *E. coli* (Mills et al. 1995). During second phase, the serotypes belonging to *S. enterica* obtained SPI-2 which *S. bongori* serotypes do not include. Pathogenic genes of SPI-2 contain an average percentage of G+C bases 41%, which is much lower than overall G+C content percentage of *S. enterica* (52%). This demonstrates that SPI-2 was acquired by S. enterica, following its discrimination from *S. bongori* (Hensel et al. 1997). Finally, the emergence of a genetic cluster (which are considered as subspecies under the current nomenclature) succeeded an important expansion of host range; warm-blooded animals, i.e. birds and mammals. In constrast, *S. bongori* along with *S. enterica* subspecies II, IIIa, IIIb, IV, VI, VII are more frequently correlated with cold-blooded organisms (Aleksic et al. 1996). This adaption to warm-blooded animals (the current reservoir of zoonotic strains of *Salmonellae*) constituted the third phase of evolution of *salmonellae* (Bäumler et al. 1998).

This evolution became possible with the addition of approximately 216 genes (Porwollik 2002), including *shdA*, which contributes to persistent colonization of intestines (Kinsgsley 2000). The target of this evolution was the invasion of intestinal mucosa and the growth of *Salmonella* over the intestinal microbiome, despite the host inflammatory resistance. Intestinal lumen, intestinal mucosa and associated lymphoid tissue were the tissues where infection developed (Tanner and Kingsley 2018).

Another finding correlated with pathogenesis of specific serotypes of subspecies I of S. enterica is the spv operon (*Salmonella* plasmid virulence operon), including *S*. Typhimurium, Cholerasuis, Gallinarum/Pullorum, Abortusovis, Paratyphi C and Dublin (Cortés et al. 2006). As these serotypes are frequently correlated with systemic infection in warm-blooded animals, it is suggested that spv operon is required for the systemic phase of salmonellosis in warm-blooded animals (i.e. *S*. Cholerasuis-pigs, *S*. Dublin-cattle, *S*. Gallinarum-poultry). However, the ability of inducing systemic disease in those species is a complex genetic mechanism, which cannot be attributed to the acquisition of just one genetic locus (Baumler et al. 1998). Moreover, genes encoding for adhesins (proteins facilitating adhesion to intestinal mucosa) may contribute to adaption of serotypes to hosts, such as *invH* (Altmeyer et al. 1993). Adhesion and invasion seem to be separate events, and this is shown by the fact that changes in genes *invA* and *invE*, affect negatively invasion but not adhesion (Ginocchio et al. 1992).

Apart from horizontal gene transfer, gene deletion or loss of genes function may affect the evolution of genome and subsequently adaption to hosts (Maurelli 2007). This is a distinct evolutionary mechanism from horizontal gene transfer (Groisman and Ochman 1997). In general, host-adapted strains are characterized by great number of pseudogenes (deactivated genes due to deletion, inserts, replacements) and chromosomal rearrangements (Matthews et al. 2010).

Moreover, some *Salmonella* serovars presented within-host evolution which facilitated the dissemination of the pathogen to other systemic sites than the intestine. This resulted in long-lasting persistence or the development of host-adapted serovars. Point mutations, horizontal gene transfer, deletions, duplications and rearrangements are genetic mechanisms contributing to the selection of traits for this purpose. This in-host evolution may occur even during infection; for example, in the presence of antimicrobials, may have impact on the bacteria and lead to exhibition of resistance genes (Tanner and Kingsley 2018). However, sometimes within-host evolution may result in extinction of the pathogen. In general, successful adaption within a specific cost demands multiple stages of genetic selection and is not easy to be conducted during a single infection.

Evolution and adaptation of *Salmonellae* in hosts, as well as in a single host, is complex and uses many genetic mechanisms, which are not completely elucidated, despite the great number of studies in this field. Further research is crucial in order to better understand the course of *salmonella* infections and the failure of treatments (Tanner and Kingsley 2018).

f) Pathogenesis

Salmonella infection may result in several syndromes, such as gastroenteritis, enteric fever, septicemia, focal infections or even asymptomatic carrier state. Specific serovars tend to cause particular syndromes (Giannella 1996). The most frequent way of entering the host is via contaminated food. However, it has been experimentally proved that many cells should be ingested in order to achieve colonization of the gastrointestinal tract, independently of inducing clinical symptoms. Individual-to-individual spread of *salmonellae* also occurs. *Salmonellae* must possess specific virulence factors in order to be pathogenic, i.e. ability of invasion in host cells, a complete lipopolysaccharide coat, ability of intracellular replication, production of toxins (Giannela 1996).

Following ingestion, bacteria colonize the ileum and colon, invade the intestinal epithelium and spread into it and lymphoid follicles. Initially, *salmonellae* bind to receptors of epithelia cell surface and then folds including the bacterial cells in vacuoles are formed (*Salmonella* containing vacuole – SVC) (Goosney et al. 1999), via a process called macropinocytosis (Frances et al. 1993). Following invasion of the mucosa, an acute inflammatory response is induced and various proinflammatory cytokines are released (IL-1, IL-6, IL-8, TNF-2, IFN-U, MCP-1, GM-CSF). Diarrhoea and acute inflammatory reaction are presented only by strains which manage to penetrate the intestinal mucosa. Diarrhoea occurs due to secretion of fluid and electrolytes by the intestines. Also, *Salmonella* strains can elaborate enterotoxin-like substances, which could induce intestinal secretion, but the exact role of these toxins in the pathogenesis of *Salmonella* enterocolitis and diarrhea has not been clarified yet. Afterwards, they replicate into the cells and spread to mesenteric lymph nodes.

Also, they may spread to the rest of the host organism, via systemic circulation, as they are absorbed by reticuloendothelial cell, which confines and controls the spread of the disease. Invasion and persistence of *salmonellae* in epithelium and lymphoid tissue of intestines are responsible for the shedding of bacteria, which lasts for 3-6 weeks (Greene 2006) and becomes intermittent gradually; in contrast to typhoid *salmonella* infections which result in well-documented persistent carrier-state (even for decades) (Gal-Mor 2019).

In the case that macrophages do not impede further spreading of the bacteria, systemic disease along with enteric fever may occur. This fact may as well lead to septicemia (Rychlik and Barrow 2005). Selective invasion and colonization of lymphoid tissue - Peyer's patches is the key factor which defines the progress of the disease (local or systemic) (Hohmann et al. 1978).

g) Clinical and laboratory findings

Clinical findings of *salmonella* infection vary depending on the number of ingested microorganisms, the immune status of the host, stress factors and concurrent infections. An unofficial categorization could be as follows: gastroenteritis, bacteremia and endotoxemia, focused infection of an organ and the asymptomatic carrier state.

Acute gastroenteritis caused by *Salmonellae* usually begins within 3-5 days of exposure or after stress with high fever, anorexia, vomiting, abdominal pain and diarrhea. The diarrhea may vary from watery to mucoid or even hemorrhagic in severe cases. Weight loss and dehydration are more obvious as the disease advances.

Heavily affected animals also exhibit paleness of mucous membranes, weakness, dehydration, cardiovascular failure, shock and icterus. More rarely, the central nervous or respiratory system is involved, with clinical signs including incoordination, posterior paresis, blindness and convulsion or cough, dyspnea, epistaxis retrospectively. In general, younger or elderly animals are more often affected (Greene 2006).

Bacteremia and endotoxemia are usually observed in very young or immunosuppressed individuals. This situation could result in death following gastroenteritis and septicemia. Still, persistent fever or paleness of mucosae, weakness, hypothermia and cardiovascular collapse could be present along with either absence or presence of gastrointestinal signs. Apart from these, organ dysfunction following thrombosis or hemorrhagic tendency could occur due to disseminated intravasculat coagulation.

Infection of a specific organ with *Salmonellae* may happen after clinical or subclinical bacteremia. This is more likely to occur in unhealthy tissues, but this does not always happen (Greene 2006). Other clinical manifestations include abortion, vaginal discharge, conjunctivitis, lymphadenomegaly.

Concerning haematologic abnormalities, nonregenerative hypochromic anaemia, lymphopenia, neutropenia with a left shift can be observed in animals with systemic disease. Neutrophilic leukocytosis is encountered in the case of carriers and animals with localized infection. Abnormality of biochemical profile (hypoproteinaemia – hypoalbuminaemia, hypoglycaemia, and moderate prerenal azotaemia) is presented only in cases of severe clinical disease (Greene 2006).

h) Pathological findings

Following the oral entry of *Salmonella* and invasion into the lamina propria, edema, macrophage and lymphocyte proliferation and polymorphonuclear engagement are observed. Lower ileum, cecum and spiral colon exhibit severe lesions, including atrophy and fusion of villi along with loss of epithelium. The bacteria penetrate into regional lymph node, resulting in macrophage and polymorphonuclear infiltration. This also happens to submucosa veins causing inflammation and thrombi. Disorders of blood circulation lead to apical necrosis, haemorrhage and fibrin exudation of the villi (fibrinous necrotic enteritis). Spread of *Salmonellae* via bloodstream or lymphatic system may provoke septicemia and damages to mesenteric lymph nodes, liver and spleen (Worley et al. 2006).

i)Diagnosis

Laboratory confirmation of *Salmonella* infection is necessary. Different diagnostic tests and biological samples may be used depending on the case (Baker et al. 2010). Microbial culture still stands as the golden standard for diagnosis. Antibody and antigen detection as well as nucleic acid amplification tests can be used despite their limitations. Nonetheless, *Salmonella* isolation from biological samples, such as the oral cavity, vomit or faeces, does not necessarily mean that this microorganism is responsible for the clinical symptoms, as many animals are asymptomatic carriers. The sensitivity of bacterial culture can be improved by cultivating multiple samples or using whole faeces instead of rectal swabs (Crump et al. 2015). Interpretation of results should be done with caution.

Many protocols and media have been used for *Salmonellae* culture, and they frequently are combinations of Rapparort-Vassiliadis Medium (Vassiliadis et al. 1972). This medium is described in the Food and Drug Administration Bacterial Analytical Manual enrichment protocol for *Salmonella*. More frequently, modified Semi-Solid Rappaport-Vassiliadis Medium is used in combination with Xylose Lysine Desoxycholate Agar (Gorski et al. 2011). Buffered Peptone Water is the pre-enrichment medium used for improving the recovery of injured *Salmonella* species prior to selective enrichment. Nowadays, a Standard Operating Procedure is used for the detection of *Salmonellae*: ISO 6579-1:2017/ Amendment 1:2020. This horizontal method can be applied to products for human consumption, animal feed, environmental specimens, and samples from the primary production stage (animal faeces, dust, swabs). This procedure aims at the detection of motile strains of *Salmonellae*.

Apart from classic culture methods, there are several serological assays which can be used for *Salmonellae* detection. These methods are not widely used for diagnostic purposes, but they can be used for epidemiological studies or screening of large populations. ELISAs and lateral flow rapid tests are commercially available although their performance varies (Burgess et al. 2014).

As rapid detection of *Salmonellae* is crucial due to public health reasons, DNA-based molecular techniques have been developed during last decades (Zhao et al. 2014), allowing characterization of strains at genetic level. These methods are faster and can be used also for serotyping of *Salmonellae*. Towards this end, several molecular methods have been proposed; at first aiming to detect or not the presence of

Salmonella spp. In 1992, Rahn et al. proposed a PCR protocol with a set of primers targeting to *invA* gene which is common among the majority of *Salmonella enterica* strains (Rahn et al. 1992). Some other primer sets have also been proposed (*oriC*, *ompC*) but those specific to *invA* have been found to perform better (Malorny et al. 2003). Additionally, molecular typing is faster and can provide with more information in a timely manner (Wattiau et al. 2011). Pulsed-field gel electrophoresis (PFGE) for the purpose of molecular typing has been used since the 1990s and is currently considered the gold standard for further discrimination. Consequently, molecular typing (luminex, arrays) and in particular Whole Genome Sequencing (WGS) is considered as the future of traditional serotyping. Nonetheless, it still remains more expensive and challenging for the majority of commercial and public health laboratories. Traditional methods of typing have certainly held their place in the reference laboratories for decades, but there are drawbacks to each typing method. These methods are time-consuming and require expensive reagents, equipment, and highly trained staff. However, due to the great amount of information collected from traditional serotyping and PFGE, it is crucial that new molecular methods produce data comparable to historical data.

j) Antimicrobial Resistance

Antimicrobial drugs are widely used by veterinary surgeons for pet animals in their care and they are administered to animals by injection (intravenously, intramuscularly, and subcutaneously), orally and topically on the skin. There are four categories of antimicrobial drugs used in veterinary medicine; inhibitors for bacterial cell wall (beta-lactam drugs, fosfomycin, and vancomycin), inhibitors for protein biosynthesis (tetracyclibnes, macrolides, aminoglycoside antibiotics), inhibitors for

DNA synthesis (4-quinolones) and inhibitors for RNA synthesis (rifampicin), inhibitors for cell membrane function (such as colistine). However, bacteria have developed resistance to every category of antimicrobial drugs since the 20st century (only a few years following their therapeutic use for humans), causing difficulties to diagnosis as well as treatment of infections (Alanis et al. 2005). This selective pressure led to evolution of several molecular mechanisms which contribute to acquisition of antimicrobials resistance (AR) by bacteria. Typically, mechanisms of AR can be classified as follows:

a) Inactivation of the antimicrobial

b) Efflux or alteration in permeability or transport of antimicrobial

c) Differentiation or replacement of the target (McDermott et al. 2003, Boerlin and Reid-Smith 2008, Foley and Lynne 2008).

All these three changes can be achieved by mutations in genes or horizontally transferred foreign genes by mobile genetic elements, such as plasmids, integrons, phage, and transposons (Frye and Jackson 2013). Moreover, the emergence of multi-drugs resistance strains (i.e. *Salmonella* Typhimurium) due to over-use raises further concerns for human health along with the One-Health approach. The degree of resistance is proportional to the total volume of used drugs (in humans, farm and pet animals) along with the method of administration (Canton and Morosini 2011). Conclusively, development of resistance is inevitable since same categories of antimicrobials are used in *salmonella* infections in animals and humans (World Health Organization 2004). According to the Directive “Food and Drug Administration Guidance 213” (FDA 2013), the reduction of antimicrobial use in animals by veterinarians (especially those considered as critical for human health) is essential for the limitation of development of resistant strains.

k) Impact of *Salmonella* spp. on Public Health

Salmonellosis is a zoonosis, which means that it is an infectious disease which is transmitted to humans from animals and vice versa. It causes diarrhea, fever, abdominal pain and may be fatal. Bovines, pigs, poultry, reptiles and other animal species serve as reservoirs of *Salmonellae* spp. *Salmonellae* are frequently found in the intestines of clinically healthy animals. Regarding food, pork or poultry meat and eggs are usually sources of *Salmonellae* spp. (Mouttotou et al. 2017). The three most commonly reported S*almonella* serovars in humans in 2018 were *S.* Enteritidis, *S*. Typhimurium and monophasic *S*. Typhimurium (1,4,[5],12:i:-) followed by *S*. Infantis and *S*. Newport (ECDC Report 2018). These five most commonly reported serovars were *S*. Enteritidis, *S*. Typhimurium, monophasic *S*. Typhimurium, *S*. Infantis and *S*. Derby (ECDC Report 2018)

Salmonella spp. remain the second most commonly reported gastrointestinal infection in the E.U., causing severe financial costs regarding hospitalization (3 billion euros a year), control programs in animals, food and feed, and breeding of animals. In a recent study, it was estimated that 93.8 (95% Confidence Interval: 61.8-163.6) million human cases of gastroenteritis and 155 000 (95% Confidence Interval: 39 000 - 303 000) deaths occur due to *Salmonella* infection worldwide each year (Majowicz et al. 2010). Rate of salmonellosis is more frequent in young children compared to older children and adults. In the E.U., mandatory notification is in force regarding the disease in 25 countries. During the years 2012-2016 statistically significant increasing trends have been observed in seven Member States (Greece, Malta, Poland, Portugal, Romania, Slovakia and Spain) (ECDC Report 2016). In 2018 and 2017, the numbers of reported human salmonellosis cases acquired in the E.U. were found to be at the same levels (ECDC Report 2018). In total, 94,203 human salmonellosis cases were reported by 28 EU MS in 2018, with 91,857 confirmed cases resulting in an E.U. notification rate of 20.1 cases per 100,000 population while one of the highest proportions of hospitalised cases were reported in our country. Based on this data, it is obvious that *Salmonella* control measures at the primary production level along with adequate laboratory capacity is essential to reduce *Salmonella* prevalence in farm animals.

Data regarding salmonellosis with or without clinical symptoms in household pets are scarce. Prevalences vary from 0 to 9% in dogs according to Marks et al. 2011. Nevertheless, several zoonotic *Salmonella* serotypes have been cultured from specimens taken from dogs, mainly without presenting clinical symptoms (Kiflu et al. 2017). Other than gastroenteritis, *Salmonella* may cause abortion, stillbirth, meningoencephalitis, respiratory distress and conjunctivitis in dogs. Dogs fed raw food diets seem to be at higher risk as the odds of shedding *Salmonella* to be 23 times greater for them than those consuming commercial diets (Finley et al. 2007). Asymptomatic carriers shed *Salmonella* intermittently, and longitudinal studies provide evidence for multiple coinfections during relatively short time periods (Hoelzer et al. 2011).

A number of medically important serotypes for humans have been found in domestic dogs, and isolation of multidrug-resistant strains has been reported (Yukawa et al. 2017). As an example, it should be mentioned that a 2003 outbreak of *Salmonella* Typhimurium among humans in New York was linked to a small animal veterinary clinic, although the index animal was not clearly identified (Hoelzer et al. 2011). On the other side, infected humans may also be a possible source of infection for their animals. In general, it is safe to comment that contacts with dogs and cats in homes, veterinary clinics and shelters clearly represent potential threats to human health. Apart from that, *Salmonella* is characterized as an occupational hazard. Consequently, good hygiene practices, environmental disinfections, strict quarantine procedures, protection of personnel, and other biosecurity measures are therefore crucial to reduce the risk wherever dogs or cats are kept in large groups or subjected to high levels of stress (i.e. in veterinary hospitals).

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CHAPTER II

2. Enteric pathogens of dogs and cats with zoonotic implications

2.1 Introduction

The relationship between humans and dogs, our first domesticated species, began in prehistoric times, some 12,000–15,000 years ago and with cats about 5,000 years ago (Morey, 1994). Our association with dogs and cats has facilitated their spread to all continents and today the worldwide dog population has been estimated to be more than 500 million. The relationship with pets has had substantial positive benefits for various segments of the population, especially children, the elderly, socially isolated, and the handicapped (Beck, 1979). Profound psychological and physiological responses have been recorded in pet owners, including a reduction in blood pressure (Wilson, 1991), reduced numbers of visits to physicians and reduced medication (Heady and Krause, 1999), improved 1-year survival after an acute myocardial infarction (Friedmann and Thomas, 1995) and reduction in the risk factors associated with cardiovascular disease.

Pet ownership is common throughout the world and in the USA 61% of households have some companion animal, 39% have dogs: 31% have more than one and more than 50 percent of families that have a dog also have children at home (Macpherson 2005). Dog, cat, and/or bird ownership in European households varies and the average is 52% for all the 17 European countries surveyed (Anon Reader's Digest Association, 1991). The number of households with pets increased from 50% in 1999 to 55% in 2005 (Dutch Council for Animal Affairs, 2006). The breakdown in the pet-owner relationship and the remarkable fecundity of dogs produces millions of unwanted stray and feral dogs, mostly in developing countries without ongoing dog population control schemes, such as Greece (Macpherson 2005).

Although the benefits of human interaction with pets are numerous, there is significant risk of transmission of pathogens from animals to humans and causing disease. These diseases are defined as zoonoses, i.e. infectious diseases naturally transmitted from animals to humans. Nowadays, the importance of zoonoses seems even more pronounced due to the increasing number of immunocompromised people who are at higher risk of zoonotic and generally infectious diseases. The risk of pathogen transmission from pet to the owner is relatively small, but it is good to take simple precautions. Therefore, veterinarians should be able to provide accurate information to immunocompromised animal owners.

The ubiquitous distribution of dogs around the world and the vast differences in their roles, from cosseted lap dogs to strays and feral dogs makes an important difference in their role in zoonotic disease transmission and our ability to prevent and control them. In countries where stray and feral dog control programs function pet dogs usually pose the most important risk for zoonotic disease. In urban areas where large numbers of companion animals occur and the number of open spaces is limited, there is a concentration of dogs in public sites, such as beaches, parks, and playgrounds which are also visited by children, for exercise and recreation. Such places become heavily contaminated by pet faeces and there is important risk of transmission of zoonotic pathogens from animals to humans (O’ Lorcain 1994, Schottler 1998). This review focuses on the transmission of enteropathogens from animal to humans.

2.2 Enteropathogens of companion animals

Although numerous enteropathogens have been detected faeces of dogs and cats, not all have the potential for transmission to humans (Bugg et al. 1999).

Consequently, this review refers mostly to the enteropathogens with high risk of zoonotic transmission and they can be classified as follows:

a) Parasites

b) Protozoa

c) Viruses

d) Bacteria

a)Intestinal Parasites

The intestinal parasites with high risk of zoonotic transmission from animals to humans are divided in the following categories:

I. Nematodes

i) *Toxocara canis/ Toxocara cati*

Recent studies have indicated that the prevalence of *Toxocara canis* and *T. cati* has decreased significantly over the last two decades (Overgaauw 1997, Bugg et al. 1999). This reduction is most likely attributable to the routine use of broad-spectrum anthelmintics by pet owners. However, in the USA, human toxocariasis (toxocaral larva migrans) has been reported to be the most common zoonotic parasitic infection acquired from pets (Schantz 1994).

The life cycle of *T. canis* is direct. People become infected when they accidentally ingest eggs containing L_2 (the second larval developmental stage), being infective, at optimal temperature and humidity, four weeks after secreted in the faeces to the environment. After ingestion, and hatching in the small intestine, the L_2 travel through the portal blood stream into the liver and lungs. Such migratory route is known as entero-hepatic-pulmonar larval migration. The second molt takes place in the lungs, the now L_3 returns via the trachea and into the intestines where the final two molts take place. This form of infection occurs regularly only in dogs of up to three months of age. Two distinct forms of disease are commonly recognised in humans: visceral larva migrans and ocular larva migrans. It has been reported that approximately 80% of dogs younger than 6 weeks had *Toxocara* present in their intestine, although often they produced negative faecal egg results (Holland et al. 1995).

The life cycle of *T. cati* is also direct and host becomes infected by swallowing of embryonated eggs of the parasite. Kittens can be infected by the colostrum or milk during nursing in their first few days of life, but there is no evidence of intrauterine infection. It should be assumed that all young animals are infected with *Toxocara* and should be treated accordingly. In suitable environments, the eggs can remain viable for months and consequently represent a significant public health risk (Overgaauw 1997).

ii) Ancylostoma/ Uncinaria

The larvae of hookworms (*Ancylostoma* and *Uncinaria*) can penetrate the skin of humans as well as the relevant canine or feline host. This condition is more common in areas of higher humidity (tropical and subtropical regions) particularly in people who have to crawl beneath buildings, sunbathers who sun bake in areas contaminated by hookworm larvae, or people who walk around barefooted (Walker et al. 1995). Recently, it has also been demonstrated that enteric infections with *Ancylostoma caninum* can lead to eosinophilic enteritis. As the worm could easily be overlooked in pathological specimens, it is probable that this condition was under-diagnosed and was more widely distributed than currently believed (Walker et al. 1995).

Life cycle of *Ancylostoma* is direct. The usual method of infection is through the skin. Infection of the host is by the larvae, not the eggs, and the larvae mature into adult worms in the intestine. Usually, the lesions are self-limiting and the intense pruritus subsides over a few weeks. However, in cases of massive infections, the larvae may penetrate into deeper tissue, leading to pulmonary or intestinal symptoms (Robertson et al. 2000).

iii) Strongyloides stercoralis

Infection of dogs and cats with *Strongyloides stercoralis* is generally less common than with other helminthes (Bugg et al. 1999). Infected animals may be asymptomatic; however, infection can also lead to severe clinical illness.

The Strongyloides' life cycle is heterogonic - it is more complex than that of most nematodes with its alternation between free-living and parasitic cycles, and its potential for autoinfection and multiplication within the host. The parasitic has a homogenic life cycle, while the free-living has a heterogonic life cycle. In the *free-living cycle*, the rhabditiform larvae passed in the stool can either molt twice and become infective filariform larvae (direct development) or molt four times and become free-living adult males and females that mate and produce eggs from which rhabditiform larvae hatch. The filariform larvae penetrate the human host skin to initiate the parasitic cycle. The infectious larvae penetrate the skin when there is contact with the soil. Free living generations that can survive in the environment for prolonged periods. In humans, there can also be auto-infection where the larvae hatch in the intestine and then penetrate the intestinal lining, leading to reinfection.

Animals may have severe diarrhoea and bronchopneumonia and the condition can easily be confused with other generalised viral diseases common in kittens and puppies. Humans can contract infection from other humans as well as dogs. If people are infected with a few worms, there may be mild intestinal signs, such as abdominal pain and diarrhoea alternating with constipation. However, when large numbers are present, the migration of the worm through the body may result in fever, liver tenderness, nausea, vomiting, weight loss and severe diarrhoea. In immunocompromised people, the parasite can lead to a disseminated, life-threatening infection (Siddiqui and Berk 2001).

II) Cestodes

i)*Echinococcus granulosus/ E. Multilocularis*

The life cycle of *Echinococcus* involves two mammalian hosts. The definitive hosts are carnivores (dogs in the case of *Echinococcus granulosus* and foxes and dogs for *E*. *multilocularis*), with adult worms present in the host's intestines. Dogs infected with *Echinococcus* pass eggs in their faeces, and the intermediate host (usually herbivores, but can also be a human) acquires infection by the accidental ingestion of these eggs (Deplazes and Eckert 2001). Unlike eggs of *Toxocara* and *Ancylostoma* or oocysts of *Toxoplasma*, eggs from *Echinococcus* sp. are immediately infective on passage. Children often become infected when playing with dogs as eggs adhere to hairs around the infected dog's anus, muzzle and paws. After ingestion of the eggs by an intermediate host, the larvae develop into cysts (hydatid cysts), hence the name cystic echinococcosis or hydatid disease. These cysts commonly develop in the liver, lungs or brain and consequently can have a serious effect on the health of infected people. *E*. *multilocularis*, the cause of alveolar hydatid disease, is an emerging infection in the USA, Western Europe and Japan. The cysts of *E*. *multilocularis* can proliferate, metastasise and invade host organs leading to potentially life-threatening consequences (Deplazes and Eckert 2001).

Under natural conditions, transmission of *Echinococcus* sp. from the intermediate host to the dog results from a predator–prey relationship. However, human activity (e.g. feeding the viscera of home-butchered sheep to dogs) can significantly modify the transmission and distribution of this parasite and has subsequently resulted in establishment of wild animal cycles (e.g. rabies vaccination through baiting has resulted in an increase in the fox population in Europe) that serve as reservoirs of infection for both cattle and sheep (Thompson 1992). The carcasses of animals shot in areas of bush close to urban areas or killed by motor vehicles, are also potential sources of infection for dogs.

ii) *Dipylidium caninum*

Dipylidium caninum, an intestinal tapeworm, can affect both dogs and cats and is rarely found in humans. Its life cycle is indirect and fleas and lice are its intermediate hosts. Gravid proglottids are passed in the faeces of the host. These proglottids rupture and eggs are released, and are ingested by larval stages of intermediate hosts. Cysticercoids form in the arthropod intermediate host after about three weeks and, when ingested by susceptible final hosts, develop into tapeworms in the intestine (Raschka et al. 1994). The host becomes infected by swallowing fleas or lice.

Humans become infected through accidental ingestion of infected fleas or other intermediate hosts. Very young children are most often affected. Symptoms are usually absent, although abdominal discomfort, diarrhoea, and pruritus may be present. With time, infections are spontaneously lost. (Robertson et al 2002).

b) Protozoa

i) *Toxoplasma gondii*

Infection with *Toxoplasma gondii* is common in humans and other animals (Cook et al. 2000). Cats are the only definitive host for this parasite, i.e. oocysts can only develop in cats. Cats excrete these oocysts 3–4 d after eating the meat of an animal containing the bradyzoites or tachyzoites of *T. gondii* (Tender et al. 2000). In a study in the USA less than 1% of cats examined have been shown to be shedding oocysts (Dubey 1994). Humans can acquire toxoplasmosis from ingesting soil contaminated by cat faeces or more frequently from eating under-cooked meat containing bradyzoites or tachyzoites (Angulo et al. 1994, Cook et al. 2000). However, a more serious clinical condition occurs when an unborn child acquires *Toxoplasma* in utero. Toxoplasmosis is also a serious disease in immunocompromised persons, particularly those with AIDS. Infection of humans has also been recorded after an organ transplant, but this is extremely rare (Robertson et al. 2002).

Although infection with *T. gondii* is common in humans, most infections occur without the presence of clinical signs. When illness does develop, the symptoms can vary from person to person. In general, the disease is more severe in unborn and newborn children of women who first are infected during pregnancy (before protective immunity is established) than in people who are infected at other times

(Cook et al. 2000). Toxoplasmosis in immunocompromised individuals can be a serious and life-threatening condition.

ii) *Giardia duodenalis*

Giardia duodenalis is the most common pathogenic intestinal parasite of humans throughout the world, with a prevalence of 2–7% (Schantz 1991). Epidemiological evidence suggest that humans are likely to be the main reservoir of human giardiasis and it is probable that direct person-to-person transmission is more important than zoonotic transmission (Schantz 1991).

However, dogs and cats can carry strains of *Giardia* which are potentially infective to humans (Hopkins et al. 1997). Consequently, zoonotic potential must be considered, especially for immunocompromised people. In a recent study in Australia, *G*. *duodenalis* was reported to be the most common enteric parasite of domestic dogs (Bugg et al. 1999). if clinical giardiasis is reported, it is usually associated with kennel or cattery situations, which are important sources of new pets for households (Robertson et al. 2000). Giardiasis can cause acute and persistent diarrhoea, abdominal pain and rapid weight loss. Therefore, treatment of *Giardia*-infected dogs and cats may be advocated, whether or not they are clinically ill, because of the potential for zoonotic transmission (Robertson et al. 2002).

iii) *Cryptosporidium*

Cryptosporidium is an important cause of diarrhoea in a variety of hosts including humans and domestic animals. In humans, infection with *Cryptosporidium* is one of the most common non-viral causes of diarrhoea (Current and Garcia 1991). The majority of cases occur in children, particularly those attending childcare centres (Hanna and Brooks 1995). *Cryptosporidium* has also been detected in dogs and cats, and these animals may represent potential sources of infection for humans (Sargent et al. 1998). Molecular studies have indicated that although *C. parvum* is composed of at least eight genotypes, only two appear to be capable of infecting immunocompetent humans (Morgan et al. 1998), but it is possible that individuals whose immune system is deficient, as in AIDS, may be susceptible to other genotypes of the parasite, including the genotype commonly found in dogs (Pieniazek et al. 1999).

Most infections of dogs with *Cryptosporidium* are asymptomatic, and clinical cryptosporidiasis appears to be most severe in the very young where the effects are exacerbated by stress, overcrowding and immune suppression (Robertson et al 2002). In humans, infection with *Cryptosporidium* has been associated with acute transient diarrhoea, but persistent infections may develop, causing severe chronic disease which may become life threatening. Fortunately, in people with a normal immune system, symptoms of cryptosporidiosis usually last only a few days or weeks, but in immunocompromised people infection can lead to a serious, life-threatening illness. Thus, with *Cryptosporidium*, early diagnosis and treatment is crucial.

c) Viruses

i) Rotaviruses

Rotaviruses are enteric pathogens causing acute watery dehydrating diarrhea in various host species, including birds and mammals. *Rotaviruses* are classified into an individual genus within the family *Reoviridae. Rotaviruses* are 70–75 nm in diameter, icosahedral, triple-layered, and nonenveloped. The genome consists of

11 segments of double-stranded (ds) RNA (Estes 2001, Kapikian et al. 2001). They are resistant to external environment.

To date, seven serogroups (A to G) of *rotavirus* have been determined (Saif and Jiang 1994). Group A *Rotaviruses* (*GARV*s) have been shown to be important cause of diarrhea in humans and a variety domesticated and captive mammals as well as poultry. Although each rotavirus serogroup has been associated with diarrhea, their distribution, epidemiology and impact shows remarkable differences in various host species. The epidemiology of *GARV*s is complicated. As with other enteric infections, *GARV*s are transmitted mainly via faecal-oral route. The stability of *GARV*s in the environment accounts for the possibility of water- or food-born outbreaks. Non-*GARV*s are considered to have considerably less public health importance. *GARV*s are not regarded as major enteric pathogens of cats and dogs but *rotavirus*-like particles have been detected at low frequency from both symptomatic and asymptomatic domestic carnivores (Marshall et al. 1984).

Although *rotaviruses* infect particular species preferentially for which they have been defined as the homologous strains, heterologous *rotavirus* infections may occur in both, natural and experimental conditions. A number of studies have also proven that under experimental conditions, *rotavirus* strains can infect and/or induce diarrhea in a heterologous animal model. Human *GARV* strains have been shown to cause disease in several newborn animals (Kapikian et al., 2001). Evidence for interspecies transmission and for genetic reassortment between human and animal rotaviruses has been continuously accumulating in the literature and, in particular, some animal species, including cats and dogs, appear to contribute frequently to the antigenic/genetic diversity found in human rotaviruses, presumably because of the close interactions between humans and these animals. Continuous epidemiological surveillance is critical for understanding the short- and long-term effects of the vaccines on rotavirus ecology and implementing future vaccine strategies.

ii) Coronaviruses

Coronaviruses (family *Coronaviridae*, order *Nidovirales*) are large (80-220nm), single stranded, positive-sense RNA viruses, which are responsible for enteric and/or respiratory disease in mammals and birds (Enjuanes et al., 2000). In dogs, a *CoV* associated with mild enteritis, *canine coronavirus* (*CCoV*), has been known since 1970s. Several *canine coronavirus* (*CCoV*) outbreaks have been reported worldwide, showing that *CCoV* is an important enteropathogen of the dog (Decaro and Buonavoglia 2008). Serological and virological investigations have demonstrated that *CCoV* is widespread in dog population, mainly in kennels and animal shelters (Naylor et al. 2001, Schulz et al. 2008).

CCoV infection is characterised by high morbidity and low mortality, as well as by a typical faecal–oral route of transmission (Tennant et al., 1991). *CCoV* is shed at high titres with the faeces of the infected dogs and its infection is restricted to the alimentary tract, leading to the onset of clinical signs typical of the gastroenteric involvement including loss of appetite, vomiting, fluid diarrhoea, dehydration and, only occasionally, death. Although it is considered rare to infect people, the increasing frequency of acute respiratory distress syndrome (SARS), reinforced the suspicion that *coronaviruses* may be responsible for causing zoonoses. There have been found changes in virulence, tissue tropisms and/or interspecies transmission of *CoVs* which occur through genetic variations in structural and/or non-structural proteins (Guan et al. 2003, Vijgen et al. 2005). In conclusion, transmission to humans can be excluded, as long as *coronaviruses* are not strictly host specific.

d) Bacteria

i) *Campylobacter* spp.

Campylobacter spp. are Gram-negative, microaerophilic, curved, motile rods. There are 37 species and subspecies in the genus, although most are thought to be nonpathogenic. Many pathogenic campylobacter species such as *C. jejuni* ssp. *jejuni* and *Campylobacter coli* are thermophilic, and thus capable of growing at 42°C. It is considered to induce diarrhoea in dogs, cats, other domestic or wild animals and humans (Marks et al. 2011).

Many studies have examined the association between diarrhea and the presence of *Campylobacter* in the faeces. A majority of these studies have found similar isolation rates in healthy and diarrheic animals (Sandberg et al 2002, Rossi et al. 2008). In dogs younger than 12 months of age, *C. jejuni* and *C. upsaliensis* had a prevalence rate in diarrheic animals over 2 times that of non diarrheic animals, but this association was not observed in older animals (Burnens et al. 1992). Experimental infection of puppies with *C. jejuni* has resulted in mild clinical disease, indicating that this organism has pathogenic potential (Brown et al. 1999). However, regarding public health, *Campylobacter* spp. are well-recognized human pathogens, and the species most commonly causing diarrheal disease in humans include *C. jejuni*, *C. coli* and *C. upsaliensis upsaliensis* (Snelling et al. 2005). *Campylobacter* spp. are potentially zoonotic from dogs to humans, and epidemiologic analyses have established a relationship between *C. jejuni* enteric disease in humans with the presence of a dog, particularly puppies in the same household (Stafford et al. 2007). Especially children and immunocompromised individuals exposed to young pets are most likely to become infected from contact with dogs or cats shedding *Campylobacter* (Marks et al. 2011). However, other sources, such as food products, are the most common means for acquisition of this pathogen (Marks et al. 2011).

ii) Salmonella spp.

Salmonellae are ubiquitous organisms that can infect or be isolated from a variety of mammals, birds, reptiles, and insects. *Salmonella* is a pathogen for dogs and cats, but many cases are subclinical. Furthermore, not all *Salmonella* strains are equally capable of causing disease and there is also the question of infectious dose.

Studies have shown that the prevalence of *Salmonella* in healthy dogs and cats can be the same as in animals with diarrhoea (Marks and Kather 2003). Prevalence of *Salmonella* in diarrheic dogs and cats ranges from 0 to 3.5% (Van Duijkeren and Houwers 2002, Hackett and Lappin, 2003) and from 0 to 8.6% (Van Immerseel et al. 2004) respectively, whereas the prevalence range for *Salmonella* in stray or shelter dogs and cats is 0–51.4% (Spain et al. 2001, Kocabiyik et al. 2006). The prevalence of *Salmonella* also has been shown to be much higher in dogs that are fed raw food diets, and *Salmonella* was isolated from 30% of the stool samples in greyhounds fed raw chicken diets (Joffe and Sclesinger 2002).

Salmonellosis is a disease of major zoonotic importance, and all *Salmonella* organisms, with the exception of those causing human typhoid fever, infect humans and animals. More frequently, foodborne outbreaks of nontyphoid salmonellosis can occur in people through contaminated products of animal origin that have been improperly prepared, stored, or handled before consumption. Feeding raw meat to dogs increases the potential risk of transmission of *Salmonella* to people. *Salmonella* infections, though, have been associated with contaminated animal food (Behravesh et al. 2010), and other studies have shown that patients with malignancy in the hematopoietic system are at increased risk of acquiring *Salmonella* induced diarrhoea (Gradel et al. 2008).

iii) Clostridium spp.

It is a Gram-positive anaerobic spore-forming bacilli. It is one of the most widespread pathogenic bacteria, and inhabits the gastrointestinal tract of humans and animals. *Clostridium perfringens* and *Clostridium difficile* are two of the most common enteropathogens which cause diarrhoea in dogs. They can cause clinical disease but they are not considered as pathogen of high zoonotic risk but it can be found in faeces of the animals. Spores can survive in faeces or decomposed tissues. Infection is more likely to be caused by food not treated properly (Jhung et al. 2008, Songer et al. 2010) or during hospitalization.

C. difficile has been identified as a cause of 10–21% of cases of diarrhea in dogs in the general population (Cave et al. 2002). *C. difficile* may be involved in some cases of acute hemorrhagic diarrheal syndrome in dogs, but questions remain because causation has not been proven. *C. difficile* can be found in 0–58% of healthy, nondiarrheic dogs and cats, particularly young animals and dogs that visit human hospitals (Marks et al. 2002, Clooten et al. 2003). The risk of zoonotic transmission currently is unclear. However, because *C. difficile* is an important human pathogen and the strains of *C. difficile* that infect dogs often are indistinguishable from those found in people with CDI (*Clostridium difficile* infection) it is prudent to consider *C. difficile* as potentially zoonotic (Arroyo et al. 2005, Weese et al. 2010).

C. perfringens is one of the most widespread pathogenic bacteria, and inhabits the gastrointestinal tract of humans and animals. It is cultured from more than 80% of diarrheic and nondiarrheic dogs (Weese et al. 2001), whereas the prevalence of *C. perfringens* in healthy cats appears to be lower than that in dogs, with isolation rates ranging between 43 and 63% (Marks et al. 2011). *C. perfringens* enterotoxin (CPE) is detected in up to 34% of diarrheic dogs (Weese et al. 2001). Animals may be a reservoir for transmission of the pathogen among species.

iv) Escherichia coli

Escherichia coli are pleomorphic Gram-negative, nonspore-forming rods belonging to the family *Enterobacteriaceae*. *Escherichia coli* are part of the normal intestinal microflora, but can be associated with gastroenteritis in the presence of bacterial virulence factors and impaired local or systemic immunity.

Enteropathogenic strains of *E. Coli* (*EPEC*) have been isolated from human patients and dogs live in the same house (Rodrigues et al. 2004). In a more recent study, faeces from 70 cats with diarrhea and 230 without diarrhea and 15 enteropathogenic strains were found in 14 of them, whereas none enterotoxigenic *E. coli* (*ETEC*) or enterohemorrhagic *E. coli (EHEC)* (Morato et al. 2009). Those strains include serotypes known as human pathogens. Therefore, it is likely some of these strains which are excreted in faeces to be transferred from dogs and cats directly or indirectly to humans. However, transmission from pets to humans of *E. coli* should not be overlooked and should be seriously considered in cases of disease outbreaks (Marks et al. 2011).

v) Yersinia enterocolitica

Yersinia enterocolitica is a species of gram-negative coccobacillus-shaped bacterium, belonging to the family *Enterobacteriaceae*. It can survive in freezing conditions and it is characterized by global distribution, as it has a broad animal reservoir and it can also be found in the environment (Wang et al. 2000). It has been isolated in faeces of healthy dogs and cats.

Domestic animals excrete *Yersinia enterocolitica* in their faeces for many weeks after the infection. Therefore, dogs and cats are more likely to be a source of infection for humans, especially for children because of their close contact with animals. In a survey conducted in China from 2004 to 2008, 326 strains of *Y. enterocolitica* were isolated from people with diarrhea and proved that there is a close relationship between strains of domestic dogs and those that cause infection in humans (Wang et al. 2010). Nevertheless, the direct transmission of *Y. enterocolitica* from animals to humans has not been proven yet (Robins - Browne 1997). However, since cases of enterocolitis have been reported in people who have come into contact with infected animals; it should be considered dangerous for causing zoonoses (Wang et al. 2010).

There are other pathogens reported in the literature that may be responsible for causing zoonotic diseases such as *Helicobacter* spp, *Anaerobiospirillum* spp. but they are much less important for public health.

Epidemiological Significance

All these pathogens that were referred are likely to cause zoonotic diseases in humans and, especially in children. But the most important are:

1. *Salmonella spp*.

Despite the advances in hygiene even in developped countries, contamination by *Salmonella* spp. continue to influence people, especially kids (Sánchez-Vargas et al. 2011). Salmonellosis is very common in developing countries, especially in kids under 5 years old, while the problem is its resistance to many antibiotics (Graham 2002). A recent study of 79 cases in U.S.A. has shown that there is an association between salnonellosis and handling dry dog or cat food. Children under the age of two consisted the 49% of these cases (Behravesh et al. 2010).

2. *Campylobacter spp.*

Campylobacter spp. was found to be the most common bacterial cause of diarrhea in hospitalized human patients in Great Britain (Tam et al. 2012), while it is one of the four most common causes of infectious enteritis in Germany (Epple and Zeitz 2011). It is very likely for *Campylobacter* to infect kids of age 0-35 months, especially if they live in a house with a pet-dog (Tenkate and Stafford 2001).

3. *Rotavirus*

It is the most common cause of infectious diarrhea in young children, especially in those of age 6 -12 months (Yang and Fang 2011) and the ownership of a pet at home is a risk factor (Bellido-Blasco et al. 2007).

4. *Toxoplasma gondii*

Ocular toxoplasmosis is the most common cause of posterior uveitis and occurs mainly in developing countries, such as South America, Central America and the Caribbean and parts of Africa (Petersen et al. 2012). A study in Brazil has found 89 infected mothers and their children who had symptoms of the eyes (nystagmus, strabismus, retinal choroiditis) (Soares et al. 2012).

5. *Echinococcus granulosus/ Echinococcus multilocularis*

It is important parasitic zoonoses which is observed mostly at rural, nomadic and transhumant communities in many regions worldwide (Macpherson et al. 2003). In Europe, pet ownership and farming are risk factors (Macpherson 2005), as well as hunting (Kreidl et al. 1998). During the period 2003-2010, 235 persons were positive for echinococcosis in Poland (Wnukowska et al. 2011).

In Greece, too, zoonoses can be found and cystic echinococcosis is considered very important. It was a serious problem for public health in the 1970 and since then, there is epidemiological surveillance: strict controls on meat and management of stray animals (Sotiraki and Chaligiannis 2010). It appears that the prevalence of parasite reduced, both in humans and in animals. More specifically, human hydatidosis has decreased from frequency 14.8 per 100,000 inhabitants in the period 1967-1971 to 0.3 per 100,000 in 2008 (Sotiraki and Chaligiannis, 2010). A survey in northern Greece, in 1994, found that the prevalence of the parasite was 100% in sheep, 56.6% in cattle, 15.4% in goats, and 9.3% in pigs and during the period from 1988 to 1999 it has found 29% seropositivity for the specific IgG in human population (Sotiraki et al. 2003). However, it seems that the parasite is still endemic in Greece.

In a research in the area of Thessaloniki in 232 faecal samples of healthy dogs there was found: *Toxocara canis* in percentage 22.4%, *Toxoascaris leonina* at 1.3% and

Giardia lamplia 0.8% (Haralabidis et al. 1988). A more recent research in Serres in stool samples from 232 healthy owned dogs found that the overall prevalence of parasitism was 26%, with Toxocara canis detected in percentage 12.8%, *Giardia* spp. 4.3%, *Toxoascaris leonina* 0.7%, *Dipylidium caninum* 0.3% (Papazahariadou et al. 2007).

In a study in northern Greece on the detection of IgG and IgM antibodies to *Toxoplasma gondii*, it was found that the prevalence of the parasite was 1.25% and 1.1% in 1984, 1.05% and 0.93% in 1994, 0.85% and 0.8% in 2004, while the intrauterine infections were estimated at 90-200 cases (Diza et al. 2005). In another study it was found that 11.1% of the 486 sera of children tested, were positive in IgG antibodies against *Toxoplasma gondii* (Frydas et al. 2000). Furthermore, a research conducted in 5 pediatric clinics, showed that *rotavirus* was the primary cause of gastroenteritis in 166 of 393 children with gastroenteritis who participated, i.e. 42.3% of non-hospitalized and in 47.8% of hospitalized patients and 78.6% of cases took place duting the period from December to April (Koukou et al. 2010). Finally, a study in Crete, which lasted from 1993 to 2010 showed that 2912 stool samples from the 33,032 samples examined were positive to enteropathogenic bacteria, and *S. enterica* (42.3%), and *Campylobacter* spp. (33.6%) were the most common (Maraki et al. 2011).

2.3 Prevention

The most vulnerable groups are usually children (due to insufficient hygiene habits, geofagia) and the elderly, since their immune system is not always effective (Morgan et al. 1997). The adoption of an animal from pet shelters and pet shops is a risk factor, since these animals are more likely to be infected (Robertson et al. 2000). Also, pregnant women and immunocompromised individuals are most at risk than other population groups (Juckett 1997). Finally, it is more probable for people professionally involved with dogs or cats as breeders, veterinarians, animal nurses to be infected by a zoonotic enteropathogen (Robertson and Thompson, 2005).

It is certain that changing people's behavior will greatly reduce the prevalence of zoonoses (Macpherson 2005). Indeed, it is very challenging effort and can last decades to produce a result. However, there are simple procedures that prevent the transmission from faeces via the oral route (faecal - oral route).

- Regular and preventive deworming of young dogs and cats and pregnant animals which are a reservoir with great number of parasites (Juckett 1997).
- Washing hands after playing with pets (Macpherson 2005).
- Supervision of children during playing with animals or visiting public places, where animals can be found too, e.g. playgrounds.
- Veterinarians should inform the owners about the risks of pet ownership and how they can be minimized (Schantz et al. 1983).
- Programs should be implemented to control stray animals population (Macpherson 2005).

However, it is very likely that these infections come from other sources, rather than direct contact with the animals. Contamination of food, water or the environment (gardens, parks) is the most common ways of acquiring a zoonotic infection. (Good et al. 2004, Smith et al. 2007). Nowadays, owners treat pets as family members and animals are free to wander in their home areas (Overgaauw et al. 2009). Therefore, it is necessary that people are well informed by their veterinarians about the risks that may arise from pet ownership, especially if they have a child. Finally, epidemiological surveillance is crucial in order to prevent and warn about zoonotic infections and outbreaks.

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CHAPTER III

3. Diagnostic performance of a rapid in-clinic test for the detection of *Canine Parvovirus* under different storage conditions and vaccination status

Canine parvovirus (*CPV*) of the family *Parvoviridae* is one of the main enteric pathogens in dogs, especially puppies between 4 and 12 weeks old. *CPV-2* is a small, non-enveloped icosahedral single-stranded DNA virus (Strassheim et al., 1994), related to other *parvoviruses* that infect carnivores, such as *Feline Panleukopenia Virus* (*FPLV*), *Mink Enteritis Virus* (*MEV*) and *Racoon Parvovirus* (*RPV*). *CPV* 2 has undergone mutations, resulting in recognition of subtypes *2a* and *2b*, while a new strain has also been detected in Italy (Martella et al. 2004). This variant (*CPV-2c*) now co-exists with other *CPV* types in Vietnam (Nakamura et al. 2004), Spain (health advantages), Germany, France, (Decaro et al. 2011), Portugal (Vieira et al. 2008) U.S.A. (Gates et al. 2014, Hong et al. 2007), Brazil (Pinto et al. 2012) and Greece (Ntafis et al. 2010).

The gastroenteric-associated lymphoid tissues and intestinal crypts represent the target tissues for viral replication of *CPV*. This results in haemorrhagic diarrhea, the most characteristic form of clinical disease. Its duration and severity strongly correlate with the titres of maternally derived antibodies at the time of infection. Virus is transmitted via the faecal-oral route through contact with faeces, soil or fomites that carry the virus. It is shed in the faeces of infected dogs within 4 to 5 days from exposure, throughout the period of clinical disease, and reaching high titres for up to ten days after recovery (Decaro et al 2005b).

Clinical diagnosis of enteritis induced by *CPV-2* is difficult and often indefinite. Therefore, it should be rapidly confirmed by reliable laboratory methods in order to begin therapeutic efforts as soon as possible. Rapid, in-clinic immunochromatographic assays are available for the diagnosis of *CPV* infection (Schmitz et al. 2009). Apart from that, faeces from clinically ill dogs can be tested using haemagglutination, virus isolation (Desario et al. 2005) and molecular methods (PCR or Real Time PCR) (Decaro et al. 2005a). However, in-clinic tests are still the most frequently used diagnostic tool in everyday veterinary practice, as the procedure is simple, inexpensive and timely.

The aim of the present study is to evaluate the diagnostic accuracy of Rapid Diagnostic Kit of *Canine Parvovirus*, *Coronavirus* and *Rotavirus* antigen (Quicking®) in the detection of *CPV* infection in dogs compared to a well-established PCR method. The diagnostic performance of this commercial rapid test was also assessed after examining samples under different storage conditions and samples collected from animals with different vaccination status.

A total number of 78 duplicated samples were collected from dogs with symptoms from the gastrointestinal tract compatible with parvoenteritis, such as: lethargy, loss of appetite, fever, vomiting, haemorrhagic small-bowel diarrhea, and dehydration. Specimens were collected following clinical examination and detailed recording of the medical and vaccination history of each animal. In 23 of the faecal samples, the Quicking Rapid Test was performed immediately after sample collection according to the manufacturer's instructions. The rest of the samples were placed into a sterile cotton tipped swab suitable for collection and transportation of viruses (Sigma Σ-VCM) and they were stored at -20°C pending analysis. All samples were examined both with the Quicking Rapid Test and PCR. The tests were conducted independently and the readers of PCR were blinded for the result of the other method.

The Quicking Rapid Test is a combined cassette used to differentially diagnose the presence of antigens from the three enteric viruses. The test is based on a sandwich lateral flow immunochromatographic assay. A visible T band in the corresponding testing window denotes the presence of any of the three pathogens in the sample. Regardless of the collection method, the wet swab was inserted in the included buffer tube and was stirred to ensure good sample extraction, as per manufacturer's instructions. Afterwards, three drops were placed in the sample holes of the cassette of the kit. The results were read within 5-10 minutes and were classified as positive or negative. No invalid results were observed.

To extract the viral DNA, the faecal specimens were homogenized in phosphate buffered saline (PBS) at a percentage of 10% w/v. After a brief centrifugation at high speed, 200 µl of the supernatant of each specimen were used for nucleic acid purification. The aliquots were incubated at 65°C for 10 minutes to inactivate PCR inhibitors and then they were chilled on ice (Uwatoco et al. 1995). A commercial DNA Purification kit (Thermoscientific Genomic DNA Purification Kit) was used to complete extraction from the specimens according to the manufacturer's protocol.

Conventional PCR was performed using the primer pair Hfor/ Hrev that amplifies a fragment of the capsid protein-encoding gene *CPV-2*, according to Decaro et al. 2005a, with slight modifications. These primers yield a product of 630 base pairs. Each 50 µL reaction mixture contained PCR buffer 1X (KCl 50 mM, Tris–HCl 10 mM, pH 8.3), MgCl2 2 mM, 200 mM of each deoxynucleotide, 1 µM of each primer, 2 U of DNA Polymerase (Thermoscientific Maxima Hot Start *Taq* DNA polymerase) and 10 ml of template DNA. The thermal conditions of this protocol initially indicate an activation of Hot Start *Taq* DNA polymerase at 94°C for10 minutes. Following this step, 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min and polymerization at 72°C for 1 min, and finally an extension at 72°C for 10 min. Following PCR, electrophoresis was performed using 8 µL of the PCR products in a 2% Tris acetate-

EDTA-agarose gel. Product sizes were determined using a 100 bp molecular weight ladder.

The sensitivity, specificity and negative likelihood ratio (NLR) as well as the significance of the differences between sensitivities obtained among groups were calculated using commercial software (Calc v. 12.3.0.0 - MedCalc Software, Ostend, Belgium). Also the Kappa statistic was estimated to determine the agreement between the two methods. Kappa value of 1 indicates absolute agreement, whereas a k value of 0 indicates that agreement occurs due to chance agreement. In general, Kappa values higher than 0.6 indicate a good level of agreement. In this study, k-values were calculated using commercial software (Graph Pad Prism v.6-Graph Pad Inc., San Diego, California).

The results of both methods used per group are analytically presented in Table 3. All samples were also negative for the other two pathogens of the rapid diagnostic test.

Table 3. Results of the immunochromatographic test and PCR per group tested

SAMPLES	IMMUNOCHROMATOGRAPHIC TEST		PCR	
	Positive	Negative	Positive	Negative
Total of Samples	16	62	72	6
Vaccinated animals	0	12	12	0

Unvaccinated animals	16	50	60	6
Directly Examined	13	10	17	6

The sensitivities and the NLR of the rapid diagnostic test in the total number of samples, in samples of non-vaccinated dogs and in samples tested directly after collection, are presented in Table 4.

Table 4. Sensitivity along with the Negative Likelihood Ratio (NLR) values for each separate group and comparison of proportions

SAMPLES	SE	95% CI	NLR	95% CI
Total of Samples	$22.22\%^{a}$	13.27%-33.57%	0.78	0.69-0.88
Vaccinated animals	0^{b}	-	-	-
Unvaccinated animals	$26.67\%^{a}$	16.08%-39.66%	0.73	0.63-0.85
Directly Examined	$76.47\%^{c}$	50.1%-93.04%	0.24	75.12%-100%

a, b, c: Figures with different superscripts differ significantly (P<0.005)

The specificity of the test was 100% in any case. (95% CI: 0.69% to 0.88 %). The Kappa value between the methods in the total number of samples, in different vaccination status and under different collection methods are presented in Table 5.

Table 5. The K-value estimation between the in-clinic assay (Quicking®) and the established PCR method as well as the strength of agreement corresponding to each calculation for the three groups

SAMPLES	AGREEMENT	
	K-value	Strength of agreement
Total number of samples	0.028	Poor
Unvaccinated animals	0.038	Poor
Samples examined directly	0.203	Fair

Canine Parvovirus still represents a major cause of morbidity and mortality in puppies, despite widespread vaccination. A rapid and definitive diagnosis of *CPV-2* infection is crucial, especially in spaces overcrowded with dogs (kennels, shelters and veterinary hospitals) in order to isolate infected animals, start treatment and prevent further spread of the virus. Commercial in-clinic rapid tests are the only assays that allow a quick and low-cost diagnosis of *CPV* in faeces of dogs. According to recent studies, these tests seem to also detect the most novel *CPV- 2c* variant (Decaro et al., 2010, Markovich et al., 2012, Decaro et al., 2013).

Immunochromatographic assays are less sensitive compared to laboratory methods, as they require a significant quantity of viral antigen to produce a visible band. Additionally, the interpretation of the result depends on the subjectivity of the operator, especially when the virus quantity is low. Consequently, more sophisticated laboratory techniques should be used for the reliable diagnosis of parvoenteritis, such as haemagglutination (HA), virus isolation (VI) and the polymerase chain reaction. These techniques can only be carried out in specialized laboratories and by trained personnel.

Despite the short time required for the performance of the test (about 4 hours), the HA assay is not frequently used because of the demand for fresh porcine erythrocytes (Desario et al. 2005). Additionally, some *CPV* strains do not have any haemagglutination activity (Parrish et al. 1988, Cavalli et al. 2001). On the other hand, virus isolation is time-consuming (requiring an incubation period of 5-10 days) and labor-intensive (demand for cell cultures, specialized staff, additional testing by HA). Although both methods are highly specific (Schmitz et al. 2009), they are characterized by low sensitivity probably due to the presence of high antibodies titres in the intestinal lumen which may bind to virions and lead to false negative results (Desario et al. 2005). Furthermore, they are less likely to detect *CPV-2*, during the late stage of the infection, when the amounts of the virus shed in faeces are significantly reduced.

It has been proved that molecular methods are more sensitive than traditional techniques, (Decaro et al. 2005a, Desario et al. 2005). The high sensitivity of PCR allows the detection of animals shedding *CPV* at low titres in the faeces. This can result in the timely introduction of measures for prophylaxis and prevention from epizootics in overcrowded spaces. Moreover, conventional PCR provides quick results, i.e.: within 6 hours (Decaro et al. 2005a). However, positive results that do not correspond to natural infection may occur due to vaccine-induced faecal shedding of the *CPV*. Modified live-virus vaccinations are very common and *CPV* vaccine persistence in the organism has not been studied thoroughly. Since, no gold standard has been established for the detection of *CPV* in faeces and PCR is the most sensitive assay, it serves as the reference method in the present study (Schmitz et al., 2009).

In our study, we compare the results between the Quicking Rapid Test and conventional PCR, in the total number of the samples, in vaccinated animals and in samples tested immediately after collection. It was demonstrated, by calculating the k-value, that there is poor agreement between the two laboratory methods when the total number of samples and non-vaccinated animals were examined. Nevertheless, it was found that there is fair agreement between the in-clinic assay and the reference method, when the samples were tested directly after the collection.

Sensitivity and specificity results suggest that a positive result almost certainly indicates the presence of the virus but negative results do not rule out the possibility of *CPV* infection. As it has been reported in previous studies, the most likely explanation for the low sensitivity of the immunochromatographic assay compared to PCR is the sequestration of viral particles by gut antibodies mainly during late stages of infection (Desario et al. 2005, Decaro et al. 2005b). Furthermore, the fact that all vaccinated animals were negative in the immunochromatographic assay and positive in PCR, could be attributed to the low vaccine viral loads that were detected by PCR but not by the in-clinic test (Desario et al. 2005). The possible presence of the new *CPV-2c* variant does not account for the low sensitivity of the rapid test, as previous studies have shown that *CPV-2c* is detected by similar immunochromatographic assays (Decaro et al. 2010).

Other statistical methods commonly used for the evaluation of diagnostic methods are positive and negative predictive values that are influenced by the prevalence of disease. Since an accurate estimation of disease prevalence is rather challenging to be made, the two above-mentioned values were omitted. Instead, Negative Likelihood Ratio (NLR) was chosen, as it does not depend on disease prevalence. In fact, a good performance of the method is demonstrated when NLR value is <0.1, while NLR >0.5 suggests a poor performance. For the total number of samples, as well as for the samples of unvaccinated animals, NLR was high, but it was adequately low for the immediately tested samples. The denominator of the equation estimating the Positive Likelihood Ratio (PLR) is zero due to specificity found 100% in all cases, leading to impossible calculation of this value.

In conclusion, the Quicking Rapid Test was evaluated as an extremely specific method but a poorly sensitive one in comparison to an established PCR method. This fact leads us to comment that negative results do not exclude parvoenteritis from the differential diagnosis, but a positive result almost certainly indicates *CPV* infection. Furthermore, the in-clinic immunochromatographic assay's sensitivity is increased when the samples are examined immediately after collection. This study confirms the findings of a previous research, which demonstrated that a similar assay produced negative results on faecal samples from vaccinated animals (Decaro et al. 2014). Consequently, the Rapid Diagnostic Kit of *Canine Parvovirus*, *Coronavirus* and *Rotavirus* antigen (Quicking ®) can be used for early diagnosis of parvoenteritis, to exclude the presence of the pathogen, but in questionable cases, faecal samples should be sent for further laboratory investigation. Apart from that, veterinary practitioners should always keep in mind that a correct diagnosis is based on a combination of history, clinical signs, biochemical parameters and positive faecal results. As *CPV* continues to be a serious lethal threat to puppies worldwide, additional research is required to further facilitate the etiological diagnosis of parvoenteritis.

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Conflict of Interest

None of the authors of this article has any financial or personal relationship with other people or Organizations that could inappropriately alter or bias the content of this work.

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CHAPTER IV

4. Environmental and demographic factors associated with the presence of *Canine Parvovirus* in diarrhoeic dogs

4.1 Introduction

Since its identification in 1978, *Canine parvovirus* type 2 (CPV-2) of the family *Parvoviridae* has been considered as a main viral agent of dogs and wild canids, causing acute enteritis with high mortality in young animals, but also affecting adults (Decaro et al. 2009). *CPV-2* is a small, non-enveloped single-stranded DNA virus, which resulted in antigenic variants, *CPV-2a*, *CPV 2b* and recently *CPV-2c*, following genetic evolution and mutations (Decaro and Buonavoglia 2012). These variants exhibit increased pathogenicity in dogs, wild canids (Miranda et al. 2017) and wider host range, as they are able to cause disease to cats and other feliforms (Mendenhall et al. 2016).

The virus is usually transmitted via faecal-oral route following contact with contaminated faeces, soil or fomites, but also via predation, scavenging carcasses, or oronasally (Greene 2006). It is shed at high titers in the faeces of infected animals within 4–5 days from exposure, throughout the period of clinical disease, and for up to ten days after recovery (Decaro et al. 2005b) and it can persist in the environment for weeks. *Canine Parvovirus* is replicated in rapidly dividing cells, which represent target for the virus; cells of lymphoid tissues, intestinal crypt epithelial cells, precursor cells in the bone marrow and myocardiocytes in puppies of less than 1 month of age (Goddard and Leisewitz 2010).

CPV is a ubiquitous pathogen which is found worldwide and it is considered as the most common viral enteric pathogen in dogs, along with *Canine Coronavirus* (Decaro et al. 2011, Decaro and Buonavoglia 2012). Several recent studies (Duque-García et al. 2017, Apaa et al. 2016, Zhao et al. 2016, Duijvestijn et al. 2016, Filipov et al. 2016, Cavalli et al. 2014, Decaro et al. 2011) have demonstrated percentage of *CPV* infection from 5.8% to 92.98%, following laboratory examination of faeces of clinically ill dogs. Risk factors associated with exposure to *CPV* include factors related to animal management, behavior and individual features of the dogs (de Almeida Curi et al. 2016). The former includes number of dogs per household, number of people per household, feeding, access to forest or villages, permission to roam freely, observed interactions between dogs and wildlife, recent dog disease or death, previous anti-rabies and multiple vaccination, anthelminthic treatment, veterinary assistance. The latter include age, sex, breed, sterilization, body condition, clinical alteration.

The domestic dog is the most widespread carnivore mammal in the world, with its population estimated to be more than 500 million, representing a conservation problem (Gompper 2014), due to negative influence on wildlife species (predation, competition, dogs being reservoir of diseases) (Hughes and MacDonald 2013). *Canine parvovirus* is among the most important pathogens transmitted from dogs to wild carnivores with negative impact over their population, along with rabies virus and *Canine Distemper virus* (Knobel et al. 2014). As an example, increase rate of the population of grey wolves was negatively affected due to puppy mortality associated with increased *CPV*-antibody prevalence in North America, demonstrating that this pathogen can be a main determinant of rate of wolf population increase and recolonization (Mech et al. 2008). So, it is obvious that dogs constantly interact directly and indirectly with wildlife, while extend and force of these interactions depend on the management, nutrition and care provided by their owners (Sepúlveda et al. 2015).

However, there is sparse information on risk factors and epidemiological parameters associated with *CPV* prevalence in dog populations in areas shared by humans, dogs and wildlife species in Greece, where all three variants of the virus currently circulate (Ntafis et al. 2010). Several studies conducted in other countries produced environmental risk mapping and spatial analysis of canine parvoenteritis using GIS, thus showing how an ecological approach can help improve our understanding of the spatial distribution of parvoenteritis (Curi et al. 2016, Zourkas et al. 2015, Ward and Kelman 2011). Monitoring and understanding of disease ecology is important for control and prevention measures because of the constant presence of free-roaming dogs, inside protected areas and their interactions with wildlife species. So, the aim of this study is to identify potential risk factors related to parvoenterits and to highlight the value of spatial analysis of such surveillance data.

4.2 Materials and methods

a) Study area

Thessaly is located in the central part of Greece and has a total area of 14·036 km2, which roughly represents 11% of the whole country. Thirty six per cent of the land is flat and 17% is semi-mountainous, whereas the remaining 45% is mountainous (Domenikiotis et al. 2005). The administrative region of Thessaly consists of four prefectures (Larissa, Trikala, Karditsa and Volos). These prefectures include 26 municipalities which are further divided in 545 municipality districts.

b) Samples collection

Dogs with clinical symptoms compatible with parvoenteritis such as: lethargy, loss of appetite, fever, vomiting, haemorrhagic small-bowel diarrhoea, and dehydration, submitted to veterinary clinics in Thessaly, were subjected to sample collection by the private practicing veterinarians. Specimens were collected following clinical examination and detailed recording of the medical and vaccination history of each animal. The samples were placed into a sterile cotton tipped swab suitable for collection and transportation of viruses (Sigma - VCM) and they were sent still frozen to the Laboratory of Microbiology and Parasitology, University of Thessaly, Karditsa Greece, pending analysis.

c) DNA extraction

To extract the viral DNA, the faecal specimens were homogenized in phosphate buffered saline (PBS) at a percentage of 10%w/v. After a brief centrifugation at high speed, 200 μL of the supernatant of each specimen were used for nucleic acid purification. The aliquots were incubated at 65◦C for 10 min to inactivate PCR inhibitors and then they were chilled on ice (Uwatoko et al., 1995). A commercial DNA Purification kit (Thermoscientific Genomic DNAPurification Kit) was used to complete extraction from the specimens according to the manufacturer's protocol.

d) Molecular Detection

Conventional PCR was performed using the primer pair Hfor/Hrev that amplifies a fragment of the capsid protein-encoding gene VP2 according to Decaro et al. (2005a) with slight modifications. These primers yield a product of 630base pairs. Each 50 μL reaction mixture contained PCR buffer 1×(KCl 50 mM, Tris–HCl 10 mM, pH 8.3), MgCl22 mM, 200 mM of each deoxynucleotide, 1 M of each primer, 2 U of DNA Polymerase (Thermoscientific Maxima Hot Start Taq DNA polymerase) and 10 mL of template DNA. The thermal conditions of this protocol initially indicate an activation of Hot Start Taq DNA polymerase at 94◦C for10 min. Following this step, 40 cycles of denaturation at 94◦C for 30 s, annealing at 50◦C for 1 min and polymerization at 72◦C for 1 min, and finally an extension at 72◦C for 10 min. Following PCR, electrophoresis was performed using 8 μL of the PCR products in a 2% Tris acetate–EDTA–agarose gel. Product sizes were determined using a 100 bp molecular weight ladder.

e) Data analysis

Univariate and multivariate logistic regression was used (statistical software R; R Core Team, Vienna, Austria) to examine associations between *Canine Parvovirus* detection and nine variables: utility (pet, work dogs and stray), age (young, adult, geriatric), gender (male or female), diet (raw, dry, homemade, mixed food), living conditions (indoors, outdoors, both), residing area (urban or rural/semi-rural), contact with other animals (dog,cats,other), deworming therapy (yes or no) and vaccination (yes or no).

f) Environmental parameters

Environmental variables were divided in three classes (climatic conditions, topography, human activities). Climate indices were derived from the WorldClim version 1.4. (Hijmans et al. 2005). Digital elevation model (altitude) was extracted from CGIAR-CSI GeoPortal (http://srtm.csi.cgiar.org/Index.asp). Hydrological data were extracted from HydroSHEDS (https://hydrosheds.cr.usgs.gov/). Wind speed was downloaded and formatted from the Hellenic Regulatory Authority for Energy www.rae.gr). Human development (municipalities/district/community) were downloaded from the Greek National Spatial Data Infrastructure and (http://www.geodata.gov.gr). Land uses and human population density were derived from (European Environment Agency, Copenhagen, Denmark [http://www.eea.europa.eu/data-and-maps]). The normalized difference vegetation index (NDVI), was extracted from the Copernicus European earth monitoring program (http://www.copernicus.eu) (FDC, Vincennes, France). To create environmental layers (n = 34) for the analysis, we used ArcGIS 10·1 GIS software (ESRI, Redlands, CA, USA). These data sets were converted to a common projection, map extent and resolution prior to use in the modelling program and they are presented in Table 6.

Table 6. Summary of environmental conditions used in the study.

Code	Environmental variable
clima1	Annual mean temperature (°C)
clima2	Mean diurnal temperature range(°C)
clima3	Isothermality (*clima2* / *clima7* × 100)
clima4	Temperature seasonality (standard deviation *100)
clima5	Maximum temperature of warmest month(°C)
clima6	Minimum temperature of coldest month(°C)
clima7	Temperature annual range(°C)
clima8	Mean temperature of wettest quarter (°C)
clima9	Mean temperature of driest quarter (°C)
clima10	Mean temperature of warmest quarter (°C)
clima11	Mean temperature of coldest quarter (°C)
clima12	Total annual precipitation (mm)

clima13	Total precipitation of wettest month (mm)
clima14	Total precipitation of driest month (mm)
clima15	Precipitation seasonality (Coefficient of Variation)
clima16	Total precipitation of wettest quarter (mm)
clima17	Total precipitation of driest quarter (mm)
clima18	Total precipitation of warmest quarter (mm)
clima19	Total precipitation of coldest quarter (mm)
wind2	Annual mean wind speed (m s^{-1})
Dem	Altitude (m)
waterdis	Distance from water collections (m)
farmsdis	Distance from small ruminant farms (m)
goatsden	Sheep density (animals km^{-2})
landcorine	Land use (principal)
aprndvi	April NDVI
mayndvi	May NDVI
junendvi	June NDVI
julyndvi	July NDVI
augndvi	August NDVI
sepndvi	September NDVI
octndvi	October NDVI
novndvi	November NDVI
popden	Human population density (people km^{-2})

g) Environmental Niche Model (ENM)

Maximum entropy modelling (MaxEnt software ver. 3.3.3) was used to predict the appropriate ecological niches for *CPV* positive dogs. Maxent method requires presence-only data, utilizes both continuous and categorical data and includes efficient deterministic algorithms and mathematical definitions (Phillips et al. 2006). Dogs that were *CPV* positive were used as occurrence points for the ENM procedure. The goodness of fit of the model predictions was evaluated by the mean area under the curve (AUC) of the receiver operating characteristic curve (ROC). We used the Jackknife procedure to reduce the number of environmental variables to only those that showed a substantial influence on the model.

4.3 Results

a) Dog population and management traits

Regarding the canine population, *CPV* infected dogs were found in each one of the four prefectures of Thessaly. In particular, 11 dogs in the prefecture of Trikala, 20 dogs in the prefecture of Karditsa, 23 dogs in the prefecture of Magnisia and 15 dogs in the prefecture of Larissa. Location of each *CPV* positive dog is depicted in Figure 5.

According to their lifestyle, 23.5% (16/68), 51.5% (35/68) and 25% (17/68) of the dogs used in this analysis were stray, pet and work dogs (shepherd and hunting dogs), respectively. Overall, the 58.8% (40/68) of the dogs were males. Concerning the age groups, 33/68 of the dogs were young (<1 years old), 31/68 adults (≥1 and <9 years old) and 4/68 old (≥ 9 years old), respectively.

b) Statistical analysis

Young dogs under 1 year of age had significantly higher odds to be positive fo*r Canine Parvovirus*. Also, multivariate logistic regression demonstrated that work dogs (shepherd and hunting dogs) had higher odds to be *CPV* infected compared to pet-dogs. No statistically significant association was found between *CPV* shedding and factors of gender, diet, living conditions, contact with other animals, deworming therapy and vaccination.

c) Predictive ENM for canine cases

Maximum entropy modelling (MaxEnt software ver. 3.3.3) was used to predict the appropriate ecological niches for *CPV* positive dogs. Maxent method requires presence-only data, utilizes both continuous and categorical data and includes efficient deterministic algorithms and mathematical definitions (Phillips et al. 2006). Dogs that were *CPV* positive were used as occurrence points for the ENM procedure. The contribution of environmental parameters to the model are presented in Table 7. The goodness of fit of the model predictions was evaluated by the mean area under the curve (AUC) of the receiver operating characteristic curve (ROC), as it is shown in figure 3. We used the Jackknife procedure to reduce the number of environmental variables to only those that showed a substantial influence on the model, as it is demonstrated in figure 4.

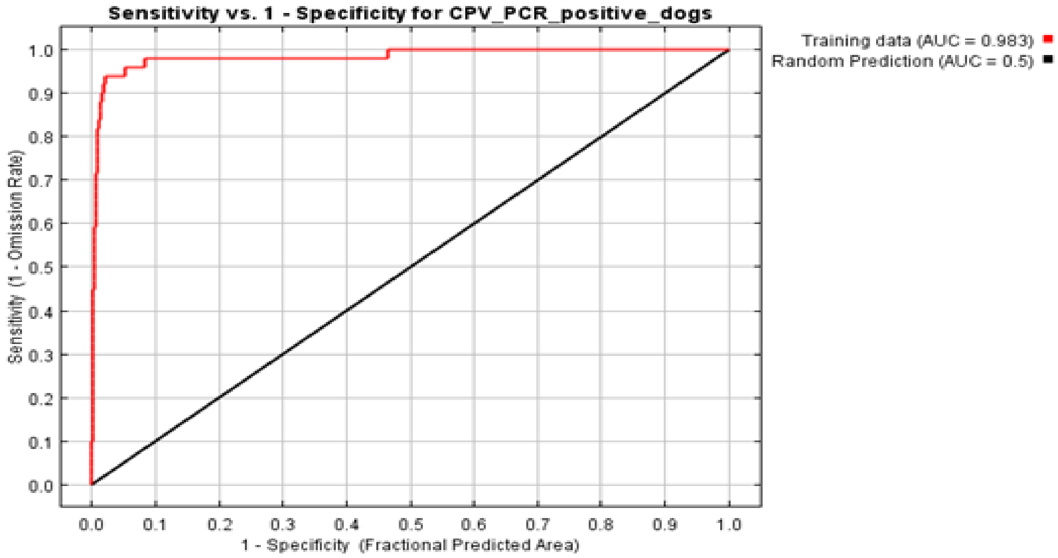


Figure 3. Depiction of the goodness of the fit of the model for *CPV* positive dogs. Area Under the Curve (AUC) value of 0.983 exceed that of random prediction of 0.5.

Table 7. Contribution of the environmental variables to the MaxEnt model.

Variable	Percent contribution	Permutation importance
goatsden	50.9	76.3
popden	27.6	1.5
landcorine	12.8	3.7
junndvi	2.6	3.8
farmsdis	1.8	0
dem	1.3	0.3
clima1	1.3	11
clima12	0.8	2.9
waterdis	0.5	0.2
wind2	0.2	0.1
aprndvi	0.1	0.1
mayndvi	0	0

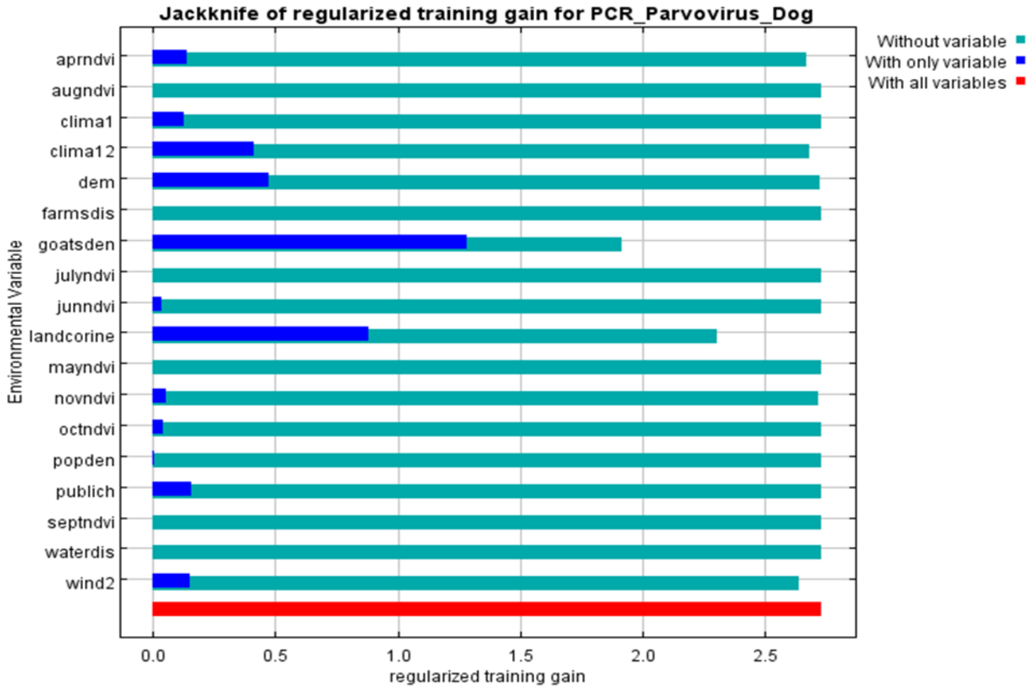
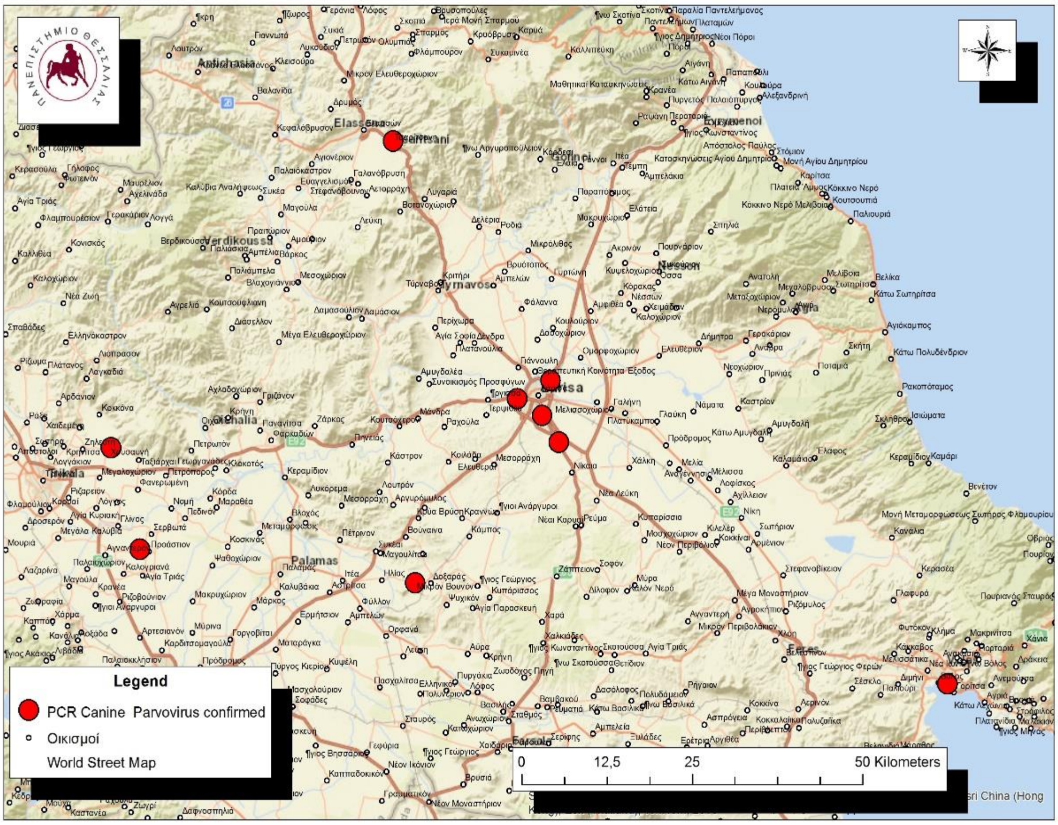


Figure 4. Depiction of Jackknife procedure for *CPV* positive dogs. Environmental variables with substanstial contribution to the model: livestock density, land uses (landcorine), human population density.

The environmental variable with highest gain when used in isolation is livestock density (goatsden), which therefore appears to have the most useful information by itself. The environmental variable that decreases the gain the most when it is omitted is livestock density (goatsden), which therefore appears to have the most information that isn't present in the other variables. Land uses and specifically the category: discontinuous urban fabric (most of the land is covered by structures, buildings, roads and artificially surfaced areas are associated with vegetated areas and bare soils, which occupy discontinuous but significant surfaces) agricultures and agroforestry formations. Also, human population density are the most significant environmental parameters as factors associated with *CPV* shedding according to the maximum entropy model.



GIS:Wildlife Ecology, Zoonoses & GIS Research Team Laboratory of Microbiology and Parasitology, Veterinary Faculty University Of Thessaly Greece

Figure 5. Map of dogs found positive to *CPV.*

In accordance with the results presented, a *CPV* potential distribution in Thessaly is depicted, following MaxEnt analysis with *CPV* PCR positive used as presence data (Figure 6).

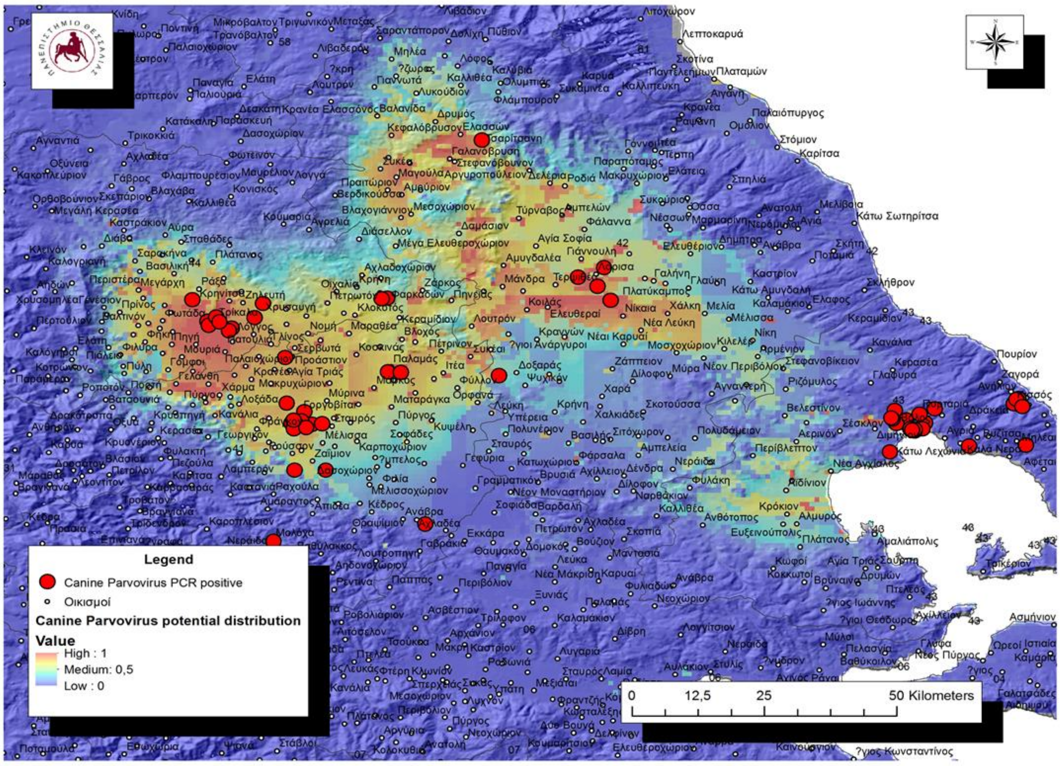


Figure 6. Potential distribution of *Canine Parvovirus* in Thessaly.

4.4 Discussion

In this study, we used ENM from MaxEnt (Phillips et al. 2006) in order to identify the environmental variables related to parvoenteritis cases in dogs, to recognize suitable areas for potential occurrence of parvoenteritis in the region of Thessaly, Central Greece and multivariate statistical analysis to identify associations between several factors and *CPV* infection. Frequent contact with domestic dogs increases the exposure and risk for disease spill over for wild carnivores (Prager et al. 2012, Woodroffe et al. 2012). Moreover, there is a scarcity of studies including GIS analysis combined with molecular diagnosis in order to associate parvoenteritis with environmental parameters. Previous studies have mostly tried to compare prevalence of exposure to canine parvovirus using serological methods between rural and urban regions (Acosta-Jammett et al. 2015, Zourkas et al. 2015) or pathogen exposure in regions where dog-wildlife interaction occurs (Curi et al. 2016).

The GIS analysis revealed that livestock density is an important environmental parameter which has significant impact on the presence of canine parvoenteritis. This fact indicates that the presence of canine parvoenteritis in dogs is associated with livestock density, i.e in regions where there is increase of livestock population. Thessaly is a Region which includes urban centers such as Larissa or Volos as well as rural regions of crops, fields and farm animal holdings. Using ENM from MaxEnt has revealed that canine parvoenteritis is more common in regions of increased livestock populations, such as rural regions which is in accordance with findings from previous studies (Zourkas et al. 2015, Belsare and Gompper, 2013; Orozco et al. 2014). Several studies have supported that dogs that live in rural areas or in areas of socio-economic disadvantages are at greater risk for suffering from the disease (Zourkas et al. 2015, Brady et al. 2012). People who live in rural areas, such as farmers or livestock breeders, tend to have shorter lifespans, more difficulties making ends meet or limited access to medical facilities, education opportunities (Eurofound 2014), especially in less favoured mountainous areas which are characterized by low revenues, aging population, low educational and cultural and lack of infrastructure (Harpa et al. 2016). This may have a negative impact on the veterinary care, diet and housing of dogs in those areas (Brady et al. 2012). The increased livestock density also implies more food available for terrestrial predators, such as foxes, a possible wildlife reservoir of the virus, resulting in increased circulation of *CPV* in specific areas around villages or livestock farms. It is well documented that *CPV* can persist in environment for long time and could be transmitted indirectly (McCaw and Hoskins 2006- Greene).

Nevertheless, a recent study demonstrated that it could be carried and transmitted by vectors, such as flies or other insects which live in abundance around livestock farms (Bagshaw et al. 2014). In such environments with increased livestock density, shepherd, owned or stray dogs also tend to roam and move more freely. For instance, in a recent study, the probability of wolf exposure to *CPV* was proved to be correlated with density of farms in a buffer zone around the place where the wolf was found, indicating that rural dogs might be the origin of *CPV* infecting wolves (Millán et al. 2016). This transmission to wolves may occurred due to scent communication (Llaneza et al. 2014, Gorman 1990), prey upon dogs (Cuesta et al. 1991), predation or scavenging (Allison et al. 2014).

Apart from that, after the recurrence of population numbers of large carnivores in Greece as in whole European continent (Chapron et al. 2014), livestock breeders usually own a large number of shepherd dogs which may interact directly or indirectly with wildlife carnivores (bears, wolves, foxes) for the protection of their herds. The same practice is followed by hunters who keep large numbers of hunting dogs, especially those involved in wild-boar hunting. This kind of dog owners tend to neglect vaccination or other obligations (such as deworming, regular visits to veterinarians), maybe due to the increased cost (economic crisis) or indifference (Millán et al. 2009) Moreover, in the present study, it was also found that *CPV* infection of dogs is associated with human population density, according to ENM from Max Entropy Model. This is a reasonable finding as increased human population usually implies an increased population of dogs which means that population of dogs depicts the distribution of human population (Gompper 2014, ACAC 2010). Apart from that, the presence and activities of humans result in increased rate of contacts between dogs and may result in increased rates of infection or easier circulation of the pathogen (Acosta-Jammett et al. 2015). In these areas of higher human population, there is also abundance of abandoned or stray dogs which try to feed on uncollected garbage and opened garbage bags or leftovers and dog food (Guilloux et al. 2018). During this study, it was also found that *CPV* infection is associated with land uses and specifically with discontinuous human fabric, i.e. suburban, rural and agroforestry land use, with suburban land use having the greatest contribution to the model. This comes in accordance with the previous finding of association between *CPV* infection and livestock density, as some people tend to keep few farm animals in suburban areas (for instance chickens or sheep) probably for private use. Also, in suburban areas, there is also increased presence of stray or freely-roaming dogs along with wildlife terrestrial predators (foxes, wolves) and it is easier for the virus to exist and circulate in the environment and spill-over between wild and domestic carnivores (Millán et al. 2016)

Finally, statistical analysis using multivariate logistic regression revealed that young age is associated with canine parvoenteritis which is supported in several studies (Zourkas et al. 2015, Duijvestijn et al. 2016, Zhao et al. 2016, Cavalli et al. 2014, Ling et al. 2012, Goddard and Leisewitz, 2010) but contradicted in others (Ward and Kelman 2011). Nevertheless, the common empirical belief among veterinarians is that young puppies among six weeks and six months are more susceptible to the disease (Goddard and Leisewitz 2010). Statistical analysis exhibited a link between being a work dog (hunting or shepherd dog) and *CPV* shedding compared with pet-dogs, which is adequately explained as these dogs may roam more freely in agro-forest or rural areas, interact with wildlife directly or indirectly and they more often live in suburban or rural areas without complete vaccination programmes. Consequently, this finding which associates work dogs with *CPV* shedding is in accordance with the results of GIS analysis.

4.5 Conclusions

This study is the first effort to simultaneously investigate possible associations of *CPV* shedding and environmental parameters in diarrhoeic dogs, in Greek territory. The Maxent method, which was used in this study, requires presence-only data while uses continuous and categorical data and includes deterministic algorithms and mathematical definitions (Phillips et al. 2006). In this way, this method improved the capability of the results analysis and revealed the environmental parameters having significant impact on *CPV* infection. Multivariate logistic regression analysis also demonstrated the variables associated with *CPV* shedding, confirming the results of previous studies. The findings of the present study should be used to improve the management of health status and habits of domestic dogs, and consequently defend susceptible wild carnivores from spill-overs of the disease, although the condition of local wild carnivores regarding infections is unknown. Interventions and educational campaigns managed by veterinarians and other stakeholders should be addressed to human population of the area, including information of owners about restriction of dog movements, sterilization, and appropriate vaccination programmes. Further studies aiming to disease surveillance and identification of risk factors and environmental parameters should be implemented in populations of dogs living in proximity with wild carnivores and measures should be taken for control and reduction of disease outbreaks.

4.6 Acknowledgements

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CHAPTER V

5. First detection of *Salmonella* spp. in hares in Greece and associated environmental factors with the infection in hares and dogs

5.1 Introduction

Salmonella spp. are the causative agents of human and animal salmonellosis, which is considered as the leading cause of hospitalizations and deaths from foodborne disease in the United States, (Scallan et al. 2011), while represent the second most frequently reported gastrointestinal pathogens in Europe with a confirmed case rate of 21.9 cases per 100,000 in general population raised up to 98.15 cases per 100,000 children in 2012 (ECDC 2014). *Salmonella* infections are more often acquired through contaminated non-animal food products (Scallan et al. 2011) but also through contaminated animal food products (Gomez-Aldapa et al. 2012) as well as through direct or indirect contact with livestock (Afema et al. 2015), rodents (Cartwright et al. 2016) wild animals (Bondo et al. 2016) and pets (Hilbert et al. 2012).

As dog ownership has increased worldwide during the last decades, it could become a growing risk for old and newly emerging *Salmonella* serovars. Despite the fact that faeces of the majority of animal species, including dogs, may serve as a potential source of *Salmonella* infection to humans and even to other animals, the implication of dogs in *Salmonella* epidemiology is sporadically investigated (Sato et al. 2000, Jajere et al. 2014, Lowden et al. 2015). Dogs have been proven to sub-clinically shed the pathogen and transmit it to humans (Sato et al. 2000, Lowden et al. 2015).

The European brown hare (*Lepus europaeus*) is one of the most important game animal species in Central Europe and it is highly adaptable to different habitat types. *Salmonella* spp. – a multihost bacterial pathogen can be found in all wildlife vertebrates including European Brown Hare, usually due to exposure to human/livestock residues (Gortázar et al. 2007). Then, wildlife species may transport the pathogen back to livestock farms. Also, hunting activities have been associated with salmonellosis in humans (Renter et al. 2006) due to the handling and field-dressing of carcasses of wild animals, including brown hares.

According to recent studies, *Salmonella* spp. are sporadically isolated from European Brown Hare (Paulsen et al. 2012, Leekitcharoenphon et al. 2016) while *salmonella* serovars are isolated from both diarrhoeic and asymptomatic dogs (Amadi et al. 2017,Arsevska et al. 2017, Kiflu et al. 2017, Reimschuessel et al. 2017) worldwide. In the most recent studies, the percentages of *Salmonella* shedding dogs range from 0.82% in UK to 11.7% in Ethiopia (Loweden et al. 2015, Kiflu et al. 2017). In Greece, there is paucity of such data.

It is well documented that *Salmonella* infection typically occurs via the faecal-oral route, by consumpting contaminated food or feed, water or by direct contacts with animals (Smith et al. 2000, Berger et al. 2010, Gomez-Aldapa et al. 2012). The reservoir of non-typhoid *Salmonellae* primarily is considered to be the intestinal tract of farm animals; however *Salmonellae* may be found in wildlife species, too (Hilbert et al. 2012). Wildlife species may get infected with the bacteria by ingesting contaminated animal feed, or water, by contacting other animals or indirectly by contacting contaminated farm buildings, manure. *Salmonella* is well known for their ability of survival and persistence in water, soil, surfaces, plants (Jacobsen and Bech 2012, Liu et al. 2018).

Also, there is sparse information on environmental parameters related to *Salmonella* presence in areas shared by humans, dogs and wildlife species in Greece. In a recent study, it was presented that more *Salmonella*-positive samples of dogs submitted to the laboratory in autumn (Arsevska et al. 2017). In another study about demographic and spatial characteristics of confirmed *Salmonella* human cases, it was discussed that a possible association between summer and early autumn months and *Salmonella* infection, as the majority of cases occurred between May and October (Seixas et al. 2018). In several studies, it has been reported that human *Salmonella* cases generally peak in summer months (Collard et al. 2008, Ravel at al. 2010). The reasons of these seasonal differences are not still completely known. A possible explanation of this seasonality in cases is that they occur due to seasonal human behaviors (Ravel et al. 2010), the pattern of *Salmonella* shedding by animal reservoirs and environmental factor alterations affecting the virulence or persistence of the pathogen (Wales et al. 2007, Ravel et al. 2010).

The application of geographic information systems (GIS) has become a very useful tool in understanding dissemination of the disease and contributing to rapid and targeted public health interventions. Therefore, this study aimed to evaluate high-risk areas and provide information on the presence of *Salmonella* spp. in dogs and hares in Greek mainland.

5.2 Materials and methods

a) Samples collection

Spleen hare samples ($n = 50$) were collected by the Division of Hellenic Hunting Confederation. The samples were submitted still to the Laboratory of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Thessaly, Greece, where they were stored at −20 °C. Data on hare specimens were located in the field using handheld global positioning system (GPS) units.

Dogs (both owned and strays) with clinical symptoms compatible with gasotroenteritis such as: lethargy, loss of appetite, fever, vomiting, diarrhoea, and dehydration, submitted to veterinary clinics located in Thessaly, were subjected to sample collection by the private practicing veterinarians. 117 faecal specimens were collected following clinical examination and detailed recording of the medical and vaccination history of each animal. The samples were placed into sterile cotton tipped swab suitable for collection and transportation (Sigma - VCM) and they were kept initially in the veterinary clinics. The samples were collected for the purpose of another study. Later, they went sent to the Laboratory of Microbiology and Parasitology, University of Thessaly, Karditsa Greece, pending analysis.

b) DNA extraction

To extract the DNA, the specimens were homogenized in phosphate buffered saline (PBS) at a percentage of 10%w/v. After a brief centrifugation at high speed, 200 μL of the supernatant of each specimen were used for nucleic acid purification. The aliquots were incubated at 65◦C for 10 minutes. A commercial DNA Purification kit (Thermoscientific Genomic DNA Purification Kit) was used to complete extraction from these specimens along with hares spleen specimens according to the manufacturer's protocol.

c) DNA amplification and gel electrophoresis

Conventional PCR was performed using the primer pair which targets to an *invA* gene sequence which has been proposed for the detection of *Salmonella* spp. according to Rahn et al. (1992), which was further validated by Malorny et al. (2003) with slight modifications. The base sequences and location of the primers are cited in Table 8.

Table 8. Base sequences and location of oligonucleotide primers.

Primer	**Oligonucleotide sequence(5' – 3')**	**Location withn the *invA* gene**
139	gtgaaattatcgccacgttcgggcaa	287-312
141	tcatcgcaccgtcaaaggaacc	571-550

These primers proposed by Rahn et al. yield a product of 284 base pairs. Each 25 µL reaction mixture contained PCR buffer 1×(KCl 50 mM, Tris–HCl 10 mM, pH 8.3), $MgCl_2$ 1.5 mM, 200 mM of each deoxynucleotide, 0.5µM of each primer, 0.75 U of DNA Polymerase(Thermoscientific Maxima Hot Start Taq DNA polymerase) and 5 mL of template DNA. The incubation conditions were 95°C for 1 min, followed by 35 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s. A final extension of 72°C for 4 minutes was employed. Following PCR, electrophoresis was performed using 8 µL of the PCR products in a 2% Tris acetate–EDTA–agarose gel. Product sizes were determined using a 100 bp molecular weight ladder. A positive response was defined as the presence of a visible band at the expected size of 284 base pair, while a negative response was defined as the lack of any band at the expected size. As a positive control, DNA extracts from *Salmonella* strains cultured from canine faeces were used. These strains were obtained by the author and submitted for serotuping to the National Reference Laboratory for *Salmonella* spp., during the thesis submitted for taking an MSc diploma in Applications of Molecular Biology-Genetics-Diagnostic Biomarkers, in 2016.

d) GIS mapping

A GIS integrates hardware, software and data for capturing, managing, analysing and displaying all forms of geographically referenced information. GIS may be applied to number of disciplines. GIS has been used to visualise, quantify and analyse geographic components of health research.

e) Data Sources

Environmental variables used in the present study were divided in three categories (climatic conditions, topography, human activities). Climate indices were derived from the WorldClim version 1.4. (Hijmans et al. 2005). Altitude (elevation model) was downloaded from CGIAR-CSI GeoPortal (http://srtm.csi.cgiar.org/Index.asp). Data on municipalities, districts, communities regarding human development were extracted from the Greek National Spatial Data Infrastructure and (http://www.geodata.gov.gr). Hydrological data and wind speed were retrieved and formatted from HydroSHEDS (https://hydrosheds.cr.usgs.gov/) and the Hellenic Regulatory Authority for Energy (www.rae.gr). Data on land uses and human population density were found from European Environment Agency, Copenhagen, Denmark [http://www.eea.europa.eu/data-and-maps]). The normalized difference vegetation index (NDVI) was retrieved from the Copernicus European earth monitoring program (http://www.copernicus.eu) (FDC, Vincennes, France). To create environmental layers (n = 33) for the analysis, we used ArcGIS 10·1 GIS software (ESRI, Redlands, CA, USA). These data sets were converted to a common projection, map extent and resolution prior to use in the modelling program.

f) Environmental Niche Model (Maxent)

In the present study, maximum entropy modelling (MaxEnt software ver. 3.3.3) was used to predict the appropriate ecological niches for *Salmonella* positive dogs and hares. Presence-only data are used in Maxent method while both continuous and categorical data can be used. Efficient deterministic algorithms and mathematical definitions are included in this method. (Phillips et al. 2006). Dogs and hares that were *Salmonella* positive were used as occurrence points for the ENM procedure.

In this study, initially all available environmental parameters were used (Table 9). It was noted that two groups of parameters with great difference concerning their percent contribution to the prediction procedure. Consequently, the parameters with percent contribution under 2% were eliminated from the last model used to predict the potential areas of species distribution. The goodness of fit of the model predictions was evaluated by the mean area under the curve (AUC) of the receiver operating characteristic curve (ROC). Jackknife procedure was used to reduce the number of environmental variables to only those that showed a substantial influence on the model. According to Ceccarelli et al. (2015) we repeated the test with the Jackknife test until all the remaining variables have a positive effect on the total gain.

Table 9. Summary of environmental conditions used in the study.

Code	**Environmental variable**
clima1	Annual mean temperature (°C)
clima2	Mean diurnal temperature range(°C)
clima3	Isothermality (*clima2* / *clima7* × 100)
clima4	Temperature seasonality (standard deviation *100)

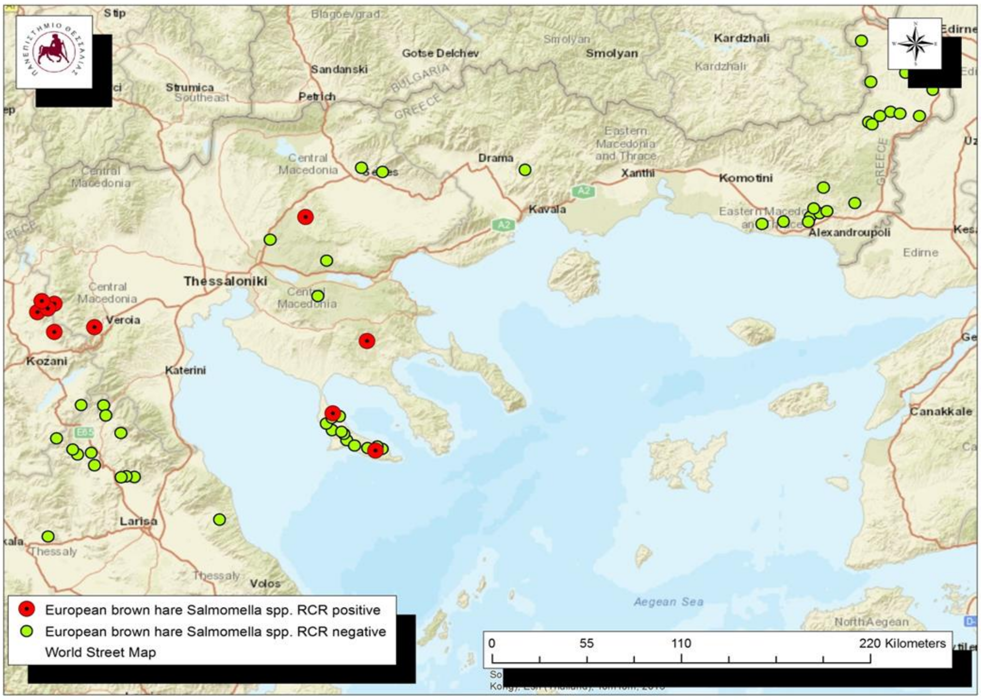
clima5	Maximum temperature of warmest month(°C)
clima6	Minimum temperature of coldest month(°C)
clima7	Temperature annual range(°C)
clima8	Mean temperature of wettest quarter (°C)
clima9	Mean temperature of driest quarter (°C)
clima10	Mean temperature of warmest quarter (°C)
clima11	Mean temperature of coldest quarter (°C)
clima12	Total annual precipitation (mm)
clima13	Total precipitation of wettest month (mm)
clima14	Total precipitation of driest month (mm)
clima15	Precipitation seasonality (Coefficient of Variation)
clima16	Total precipitation of wettest quarter (mm)
clima17	Total precipitation of driest quarter (mm)
clima18	Total precipitation of warmest quarter (mm)
clima19	Total precipitation of coldest quarter (mm)
wind2	Annual mean wind speed (m s^{-1})
dem	Altitude (m)
waterdis	Distance from water collections (m)
farmsdis	Distance from small ruminant farms (m)
goatsden	Sheep density (animals km^{-2})

landcorine	Land use (principal)
aprndvi	April NDVI
mayndvi	May NDVI
junendvi	June NDVI
julyndvi	July NDVI
augndvi	August NDVI
sepndvi	September NDVI
octndvi	October NDVI
novndvi	November NDVI
popden	Human population density (people km^{-2})

5.3 Results

European Brown Hare

10 hares out of 50 (20%) were found to be positive, as it is depicted in Figure 7.



GIS:Wildlife Ecology, Zoonoses & GIS Research Team Laboratory of Microbiology and Parasitology, Veterinary Faculty University Of Thessaly Greece

Figure 7: Map of hares found to be *Salmonella* positive.

Predictive ENM for cases of hares

The contribution of the environmental variables to the MaxEnt model analyzed in this study, concerning hares, is shown in Table 10. The environmental variables with the highest contribution for hares being positive to *Salmonella* are precipitation seasonality, precipitation of the coldest quarter of the year, the minimum temperature of the coldest month, livestock density and land uses.

The goodness of fit of the model predictions is demonstrated in Figure 8. Environmental variables with positive effect on the total gain following Jackknife of regularized training gain are depicted in Figure 9. Regularized training gain is 1.406, training AUC is 0.973 and unregularized training gain is 2.198.

Table 10. Contribution of the most important environmental variables to the MaxEnt model.

Variable	Percent contribution	Permutation importance
clima15	49.3	48.6
clima19	15	11.8
clima6	12.2	7.9
landcorine	7.7	7.1
goatsden	6.4	23
dem	5	0
wind2	4.2	1.6

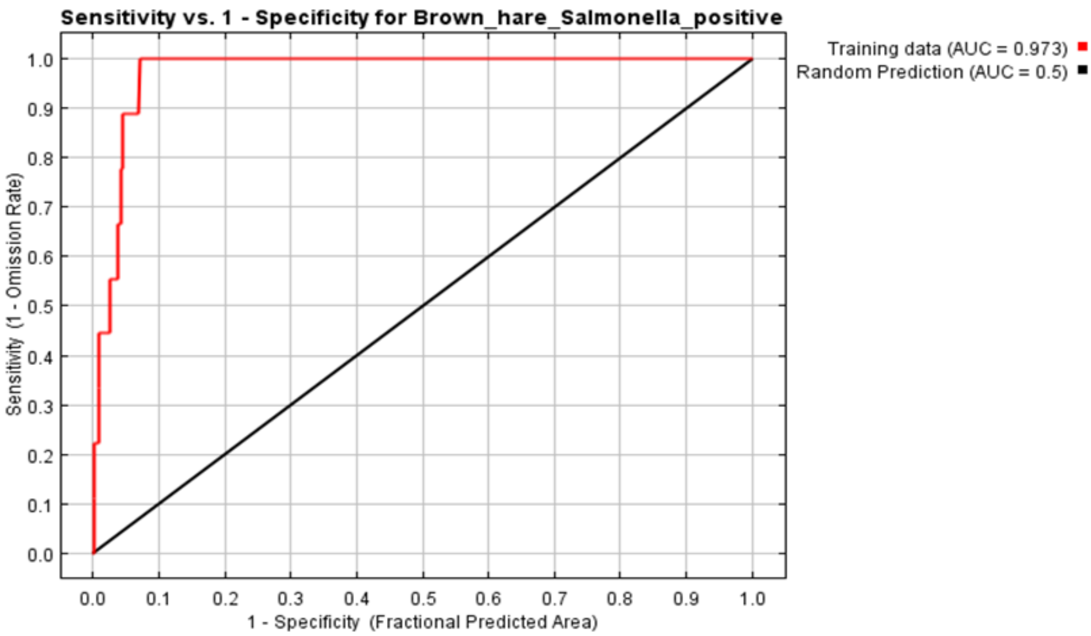


Figure 8. Depiction of the goodness of the fit of the model for *Salmonella* positive hares. Area Under the Curve (AUC) value of 0.973 exceeds that of random prediction of 0.5.

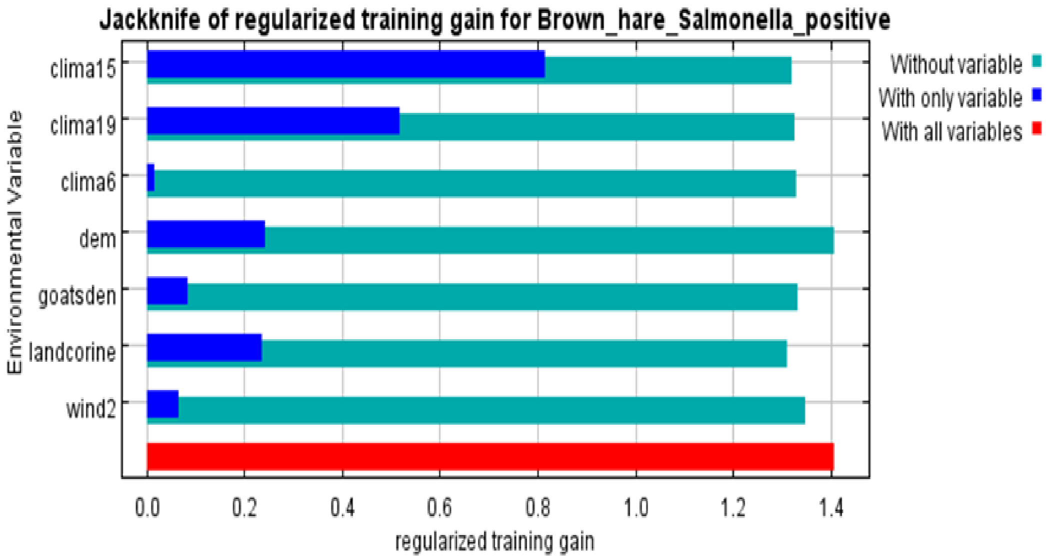


Figure 9. Depiction of Jackknife procedure for *Salmonella* positive hares. Environmental variables with substanstial contribution to the model: precipitation seasonality, precipitation of the coldest quarter of the year, the minimum temperature of the coldest month, livestock density, land uses.

In accordance with the results presented, a *Salmonella* potential distribution for hares is depicted, following MaxEnt analysis with *Salmonella* PCR positive hares used as presence data (Figure 10).

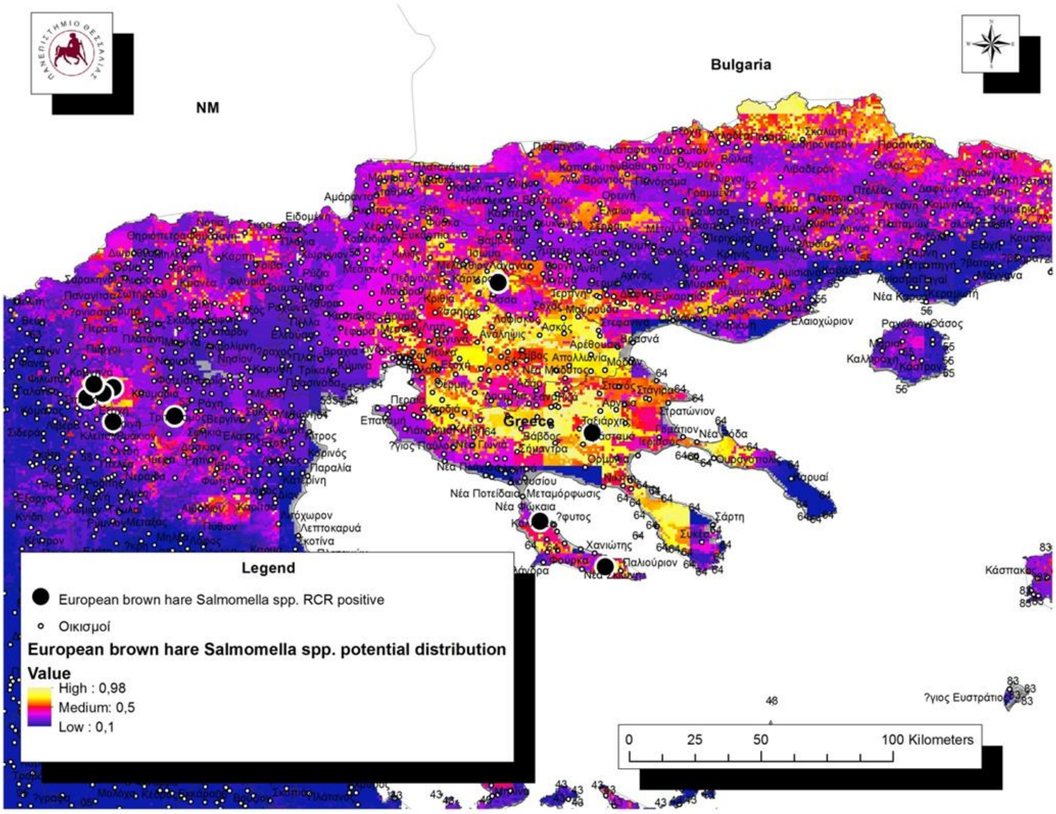
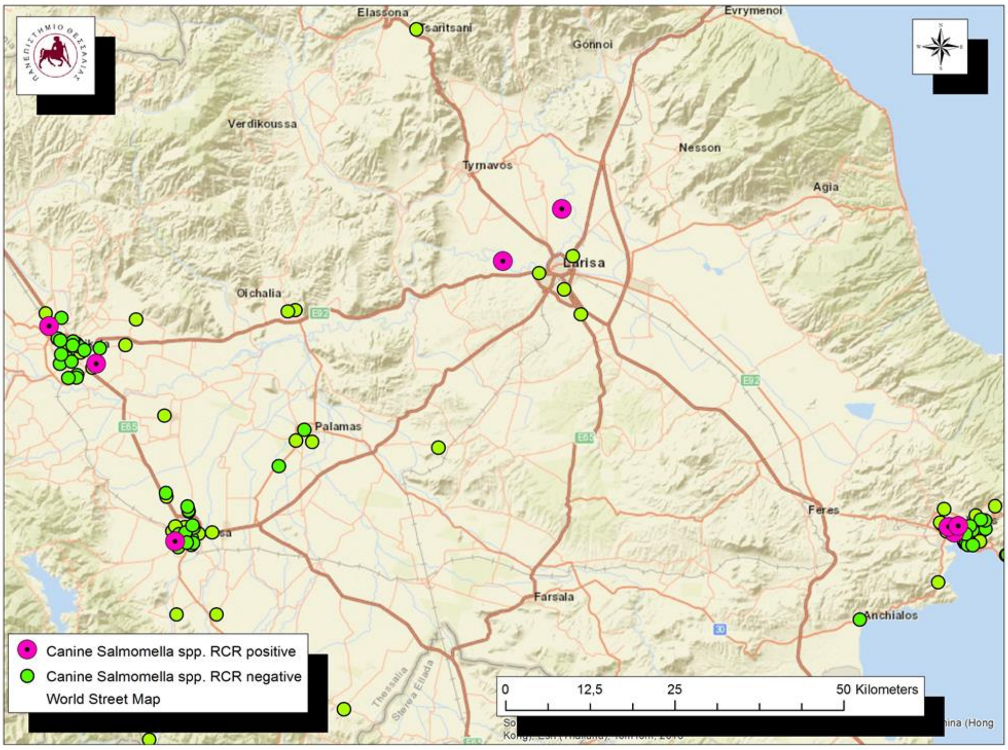


Figure 10. Potential distribution of *Salmonella* for hares using the presence data of *Salmonella* positive hares.

Dogs

8 out of 117 dogs (6.8%) were found to be *Salmonella* positive, as it is depicted in Figure 11.



GIS:Wildlife Ecology, Zoonoses & GIS Research Team Laboratory of Microbiology and Parasitology, Veterinary Faculty University Of Thessaly Greece

Figure 11. Map of dogs found to be *Salmonella* positive.

Predictive EMN for cases of dogs

The contribution of the environmental variables to the MaxEnt model analyzed in this study, concerning dogs, is shown in Table 11. The environmental variables with the highest contribution for dogs being positive to *Salmonella* are: the highest temperature of warmest month (which is the most important), the index concerning the percentage of green leaves of the vegetation during August (augndvi) followed by livestock density and land uses.

The goodness of fit of the model predictions is demonstrated in Figure 12. Environmental variables with positive effect on the total gain following Jackknife of regularized training gain are depicted in Figure 13. The regularized training gain is 2.765, training AUC is 0.986 while unregularized training gain is 3.871.

Table 11. Contribution of the most important environmental variables to the MaxEnt model.

Variable	Percent contribution	Permutation importance
clima5	47.7	67.1
goatsden	25.4	0.7
augndvi	12.7	3.6
landcorine	6.7	0
dem	5.3	0
clima9	1.3	28.2
popden	0.9	0.4

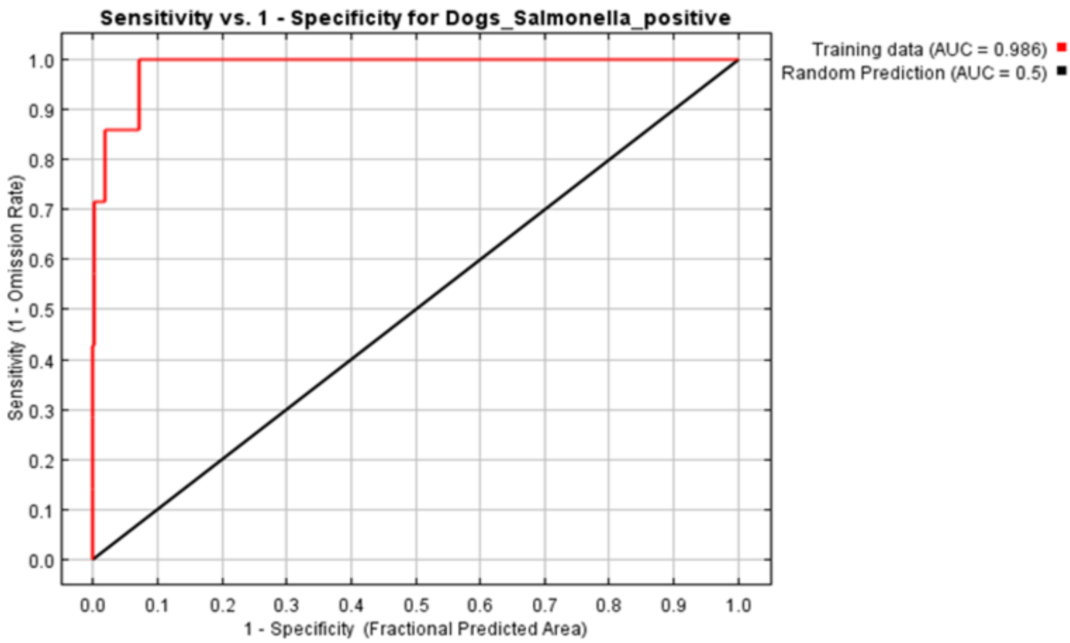


Figure 12. Depiction of the goodness of the fit of the model for *Salmonella* positive dogs. Area Under the Curve (AUC) value of 0.986 exceeds that of random prediction of 0.5.

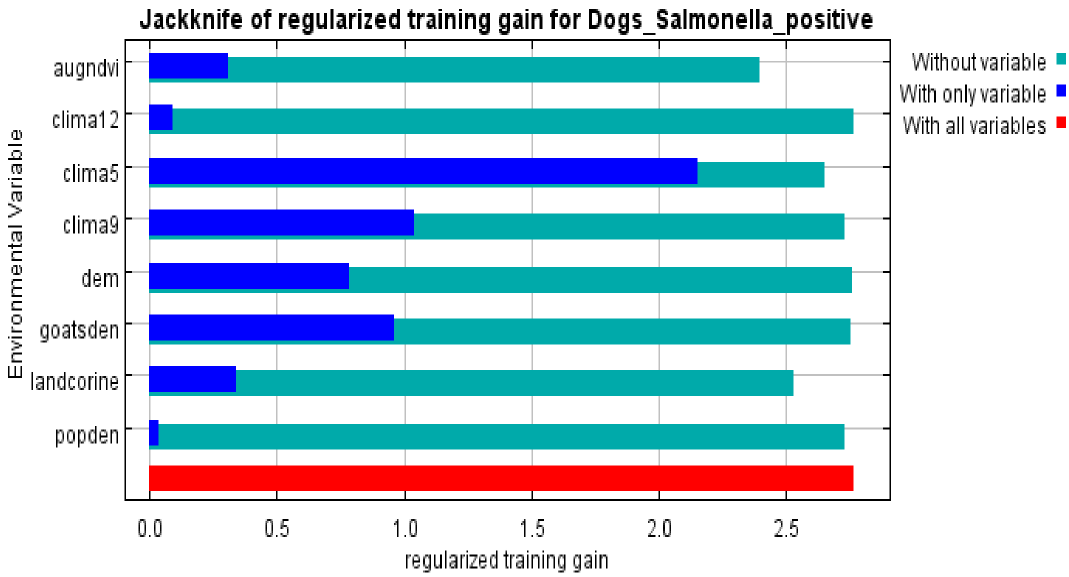


Figure 13. Depiction of Jackknife procedure for *Salmonella* positive dogs. Environmental variables with substanstial contribution to the model: the highest temperature of warmest month, the index concerning the percentage of green leaves of the vegetation during August (augndvi) followed by livestock density and land uses.

In accordance with the results presented, a *Salmonella* potential distribution for dogs is depicted, following MaxEnt analysis with *Salmonella* PCR positive dogs used as presence data (Figure 14).

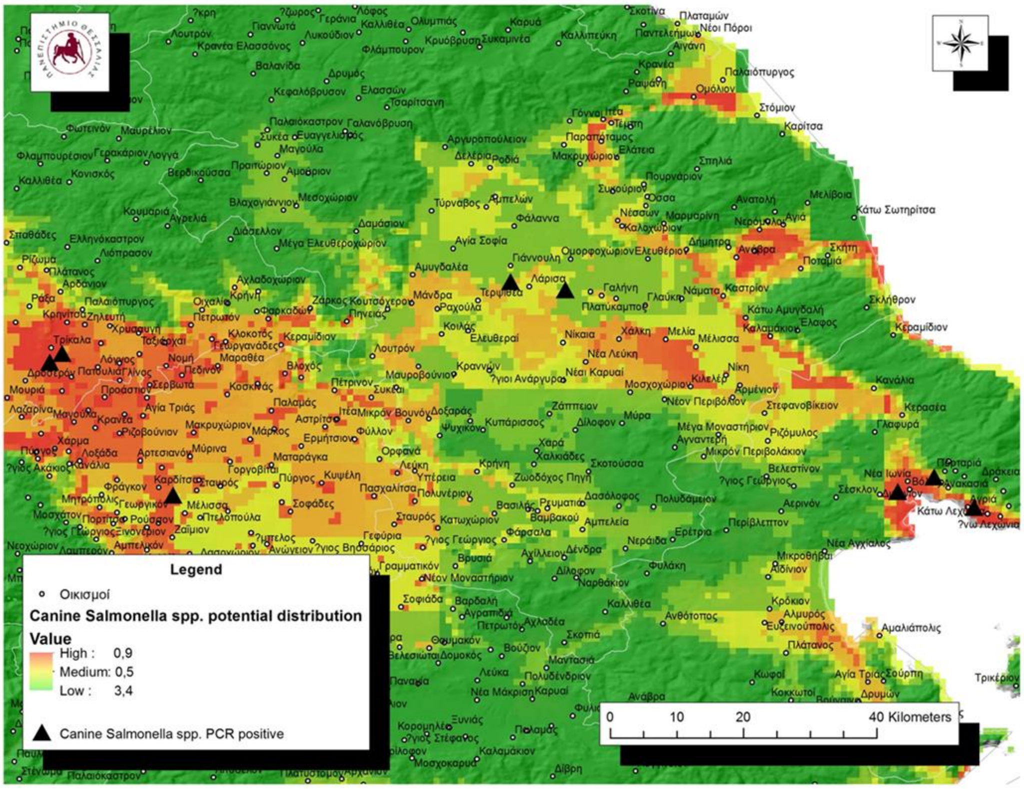


Figure 14. Potential distribution of *Salmonella* for dogs using the presence data of *Salmonella* positive dogs.

5.5 Discussion

This study provides evidence of *Salmonella* infection in a number of hares (10 specimens tested positive) and dogs (eight specimens tested positive) in Central Greece. To the best of our knowledge, this is the first time that *salmonella* presence is reported in hares in Greece. We also used ENM from MaxEnt (Phillips et al. 2006) in order to identify the environmental variables related to *salmonella* shedding in dogs and hares in Greek mainland and to recognize suitable areas for potential occurrence of salmonellosis. The analysis revealed that different environmental parameters affect the presence of *Salmonellae* in these two different species. Nevertheless, there are common environmental factors, which are correlated with the presence of the pathogen in both animal species; livestock density and land uses.

Apart from that, concerning hares the factors with the highest contribution for hares being positive to *salmonella* are precipitation of seasonality, precipitation of the coldest quarter of the year, minimum temperature of the coldest month and land uses. *Salmonellae* are not frequently isolated from European brown hares while in Greece there is paucity of such data (Broderson and Gluckstein 2014). It has been shown that some *Salmonellae* seem to be host-adapted to wild species in Europe, such as in the case of *S. enterica* subsp*. enterica* ser. Enteritidis (phage type 11) in hedgehogs (Gaffuri and Holmes 2012). Apart from hedgehogs, wild birds are a well-documented reservoir of *Salmonellae* in nature (Navarro-Gonzalez et al. 2020, MacDonald et al. 2018). Also, wild species interacting with livestock in shared, contaminated environments may acquire or transmit a *Salmonella* infection (Horton et al. 2013). Wildlife species may get infected by *Salmonella* by ingesting contaminated animal feed or water or by contacting livestock holdings, manure or directly contacting other infected animals. (Hilbert et al. 2012). These above reasons justify the presence of *Salmonellae* in hares, which is detected in Greece for the first time. The presence of *Salmonella* is well documented in water (Ho et al. 2018) and several studies have showed positive relationships between precipitation and occurrence of pathogens (Simental and Martinez-Urtaza 2008, Setti et al. 2009, Wilkes et al.2009) and this fact may explain the correlation between *Salmonella* positive hare samples and precipitation. Also, temperature and precipitation conditions may alter the nutrition of foraging and grazing animals and favor colonization of pathogens (Giannakopoulos et al. 2018). This fact along with the correlation of *Salmonella* cases with minimum temperature may be attributed to changes in dietery or other habits of hares during autumn. As it has been proved, dietery habits of hares change during spring when they rely on fat reserves during reproductive season (Schai-Braun et al. 2015).

Dogs typically acquire *Salmonella* infection from ingesting contaminated feed, including unprocessed or raw dog food, especially meat contaminated by the pathogen (Akwuobu et al. 2018) and they do not always present symptoms. This means that the faecal-oral route is the predominant way of *Salmonellae* infecting dogs. In this particular study, the dogs exhibited symptoms of diarrhoea and the environmental variables related to *salmonella* shedding in dogs included max temperature of warmest month (which is the most important), indices concerning the percentage of green leaves of the vegetation (augndvi) and then livestock density and land uses. Recent literature indicates that environmental parameters and climate may play a role in the prevalence of this microorganism along the food chain (Smith et al. 2019). In a recent research, reported human salmonellosis cases were associated with seasonal peaks in June and July and ambient temperature (Ravel et al. 2010). As dogs tend to follow their owners' dietery habits or even eat human food or leftovers, it may be suggested that the pattern of *Salmonella* infections may be closer to that of human cases, which explains the correlation of cases with the max temperature of the warmest month and the mean temperature of the warmest month. The index of increased vegetation during August is considered as a random finding in this study. Apart from that, livestock density is an anticipated finding associated with *salmonella* presence in dogs, as *Salmonellae* may be found in a variety of hosts including livestock animals, with an estimated annual number of cases ranging from 200 million to over a billion (Bierschenk et al. 2018). Some food producing animals actually serve as reservoir of certain serotypes of this pathogen, i.e. cattle or swine.

It must not be forgotten that recent extended studies of human salmonellosis have exhibited great differences in seasonality within the *salmonella* genus (Cherrie et al. 2018). Human cases of salmonellosis and their seasonality or environmental risk factors are presented under the prism of the foodborne transmission of the pathogen. Bearing in mind the fact that *salmonellae* are ubiquitous pathogens with a wide range of hosts and various ways of transmission, we can presume that the characteristics of *salmonella* infection are based on the specific habits of the host rather than the pathogen itself. Apart from that, there are differences among the serotypes of *Salmonellae* (Cherrie et al. 2018); however in our study it was not feasible to identify specific serotypes, which may elucidate further the differences of *Salmonella* strains regarding temperature.

Nevertheless, the presence of *Salmonella* spp. in both hares (a game species consumed by humans) and dogs (living in great proximity with their owners) indicates that the pathogen may be transmitted among humans, dogs, wildlife and the environment regardless of the original source or reservoir of the infection. Also, the combination of the two opposite temperatures favoring *Salmonella* presence in these two species indicate that extreme temperatures might be a stressor which could facilitate the pathogen colonization. Salmonellosis still remains as the second most frequently reported gastroenteritis in the EU (ECDC 2020) while the role of environmental factors , wildlife and weather temperature in pathogen are not fully investigated. The findings of this study highlight the need for future research in this field, aiming at better understanding the transmission routes and factors affecting this zoonotic infection. In the context of One Health and the utility of wildlife as indicators of pathogens circulating in the environment, the profound knowledge of

Salmonella serotypes-host interactions and their results are crucial for achievement of prevention.

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6.GENERAL CONCLUSIONS

The conclusions conducted by the study of this PhD thesis are the following:

- *Canince parvovirus* (*CPV*) diagnosis is still complex and indecisive, especially due to the lack of laboratory confirmation. The rapid in-clinic tests used by the majority of clinicians are characterized by various diagnostic performances and are less sensitive compared to laboratory methods. In our study, we used a specific commercial in-clinic rapid test and we compared it with a reference method of PCR in the total number of the samples, in vaccinated animals and in samples tested immediately after collection. It was found that there was fair agreement when the specimens are tested in due time following the collection; however poor agreement was reported when the total number or non-vaccinated animals were examined. Sensitivity and specificity results suggest that a positive result of the commercial test almost certainly indicates the presence of the virus but negative results do not rule out the possibility of *CPV* infection.

- Strains of *Canine Parvovirus* (*CPV*) still poses a threat as enteric pathogen for dogs, especially younger ones, despite the widespread vaccination schemes implemented for many years by clinical veterinarians. This fact is supported by the percentage of diarrhoeic samples found positive when tested with PCR method (69 dogs out of 117) during this study. Moreover, young dogs under 1 year of age had significantly higher odds to be positive for *Canine Parvovirus*. This is a reasonable finding compatible with *CPV* pathogenesis, which is confirmed in previous studies. Also, work dogs (shepherd and hunting dogs) had higher odds to be *CPV* infected compared to pet-dogs, which is an anticipated finding as these dogs wander freely in rural areas or may interact with wildlife directly or indirectly. Gender, diet, living conditions, contact with other animals, deworming therapy were not found to be correlated with *CPV* presence in this particular research.

- For the first time, association of *CPV* shedding and environmental factors is investigated in Greece. The GIS analysis displayed that livestock density along with land uses are important environmental parameters. This comes in accordance with the previous findings, a justifiable result which may be attributed to the habits of dogs living in rural or suburban areas. *CPV* infection is associated with human population density which could mean that in areas of concentrated human population, there is a higher rate of contacts among dogs and facilitation of the circulation of the pathogen.

- *Salmonella* presence was reported in a number of hares (10 specimens tested positive) and dogs (eight specimens tested positive) in Central Greece. To the best of our knowledge, this is the first time that *Salmonella* presence is reported in hares in Greece. ENM from MaxEnt was used in order to identify the environmental variables related to *Salmonella* shedding in dogs and hares in Greek mainland. The analysis revealed that different environmental parameters affect the presence of *Salmonellae* in these two different species. Nevertheless, livestock density and land uses are common environmental factors associated with the presence of *Salmonella* in those two species. The differences of environmental factors associated with *Salmonella* presence between those two animal species may indicate that the different diet and habits of the host are depicted rather than factors affecting survival and colonization of the pathogen, in this particular study.

UNIVERSITY OF THESSALY

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DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

LABORATORY DIAGNOSTIC APPROACH TO CANINE INFECTIOUS DISEASES OF WILDLIFE AND PUBLIC HEALTH IMPORTANCE

Doctoral Thesis by
Maria C. Kantere

SUMMARY

This thesis aimed to investigate specific canine infectious diseases of wildlife and public health significance by a laboratory diagnostic approach, combining laboratory techniques and environmental niche modelling, where it was considered appropriate. To this end, samples from diarrhoeic dogs were tested for the presence of *Canine Parvovirus* and *Salmonella* spp. Also, samples from European Brown hares were tested for the presence of *Salmonella* spp. only, as *Canine Parvovirus* strains prefer the carnivora as hosts. *Canine Parvovirus* is a pathogen of importance for dogs and other wild carnivore species while *Salmonella* spp. remain the second cause of human gastroenteritis cases in the EU; thus means that is of major Public Health importance.

In the first chapter of this study, the international literature was critically reviewed, and health risks associated with human animal interactions were presented. More

specifically, enteric pathogens of zoonotic risk which are transmitted by faeces of dogs and cats were referred in groups (Parasites/ Protozoa/ Bacteria/Viruses). Among them, *Salmonellae*, *Campylobacter*, *Rotaviruses*, *Toxoplasma gondii*, *Echinococcus* are of significant for many countries including Greece.

The evaluation of the diagnostic accuracy of a commercial diagnostic tool for the detection of *Canine Parvovirus* is described in the second chapter. The rapid in-clinic test was compared with an established PCR method, which is considered as the most reliable diagnostic method. 78 faecal samples were collected from diarrhoeic dogs. Implementation of vaccination within a month prior to the onset of diarrhoea was reported for 12 of the sampled dogs. The rapid diagnostic test was performed in 23 of the faecal samples directly, while the rest were placed into a sterile swab and stored at –20 ◦C. The sensitivity of the rapid diagnostic test compared to PCR in the total number of samples, in samples from non-vaccinated dogs and in samples tested directly after collection were 22.22% (95% CI: 13.27–33.57%), 26.67% (95% CI: 16.08–39.66%) and 76.47% (95% CI: 50.10–93.04%) respectively, while the specificity of the test was 100% in any case. Based on these results, it can be argued that negative results do not exclude parvoenteritis, especially in dogs with early vaccination history, but a positive result almost certainly indicates *CPV* infection. The test should be conducted directly in order to expect improved sensitivity.

In the third chapter, we aimed to investigate the presence of *Canine Parvovirus* in dogs with compatible clinical signs in Central Greece, to identify demographic and environmental factors associated with *CPV* infection. A total of 117 faecal samples were collected from diarrhoeic dogs and were tested for a fragment of *VP2* capsid gene. Multivariate logistic regression, using R program, revealed that significant factors identified were age and utility of dogs (work dogs more probable to be positive versus pet-dogs). Geographical information system (GIS) together with the Ecological Niche Model (ENM) showed that the most important findings of the study were (i) Livestock density (ii) land uses and specifically the category: discontinuous urban fabric and human population density are the most significant environmental parameters as risk factors according to the maximum entropy model.

The last chapter concerns the detection of *Salmonella* spp., an important zoonotic pathogen in dogs and European Brown Hare, which is an important game species. 50 samples (spleen tissue) from hares hunted and 117 samples (faecal swabs) from dogs were examined for *Salmonella* using a validated PCR protocol (Malorny et al. 2003). Also, a spatial analysis using GIS was conducted to investigate possible geographical distribution and environmental risk factors, associated with *Salmonella* presence. 10 hares out of 50 and 8 out of 117 dogs were found to be *Salmonella* positive. GIS analysis using Max Entropy Montel showed that risk for the exposure to *Salmonella* in hares are driven by environmental parameters; precipitation seasonality, precipitation of the coldest quarter and livestock density being the most significant. Respectively, for dogs being positive to *Salmonella* and its potential distribution, the significant environmental parameters were: maximum temperature of the warmest month, mean temperature of warmest quarter of the year, livestock density and normalized difference vegetation index (NDVI) for August. Livestock density is an environmental parameter which is shared between dogs and hares positive to *Salmonella*, indicating that there is a probable spill-over among farm animals, dogs and wildlife. This study, which is the first of this kind from Greece, suggests that the role of hares and dogs in *Salmonella* shedding and transmission deserves further elucidation.

This thesis provides evaluation of existing diagnostic methods for parvoenteritis, which is present in the canine population of the Greek mainland. It also explores the association of *CPV* presence in diarrhoeic dogs with certain demographic and environmental factors. Apart from that, the *Salmonella* presence in diarrhoeic dogs and hares is reported and associated with environmental factors which differ between the two animal species, suggesting that in this case the different habits of the hosts are depicted. The continuous surveillance of canine populations in combination with targeted wildlife species could provide information on the pathogens (especially zoonotic) circulating posing risk for wildlife, domestic animals and humans.

ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΚΤΗΝΙΑΤΡΙΚΗΣ

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Διδακτορική Διατριβή της

Μαρίας Κ. Καντερέ

ΠΕΡΙΛΗΨΗ

Ο στόχος της παρούσας διατριβής ήταν η μελέτη ορισμένων λοιμωδών νοσημάτων του σκύλου, ιδιαίτερης σημασίας για την άγρια πανίδα και τη Δημόσια Υγεία μέσω διαγνωστικής εργαστηριακής προσέγγισης, συνδυάζοντας εργαστηριακές τεχνικές και μοντέλα κατανομής ειδών, όπου αυτό κρίθηκε απαραίτητο. Για το σκοπό αυτό, δείγματα από σκύλους που παρουσίαζαν διάρροια εξετάστηκαν για την εύρεση του ιού *Canine Parvovirus* και σαλμονέλλας. Επίσης, δείγματα από ευρωπαϊκούς καφετί λαγούς εξετάστηκαν για την παρουσία σαλμονέλλας, εφόσον τα στελέχη *Canine Parvovirus* προτιμούν σαρκοφάγα είδη ως ξενιστές. Ο ιός *Canine Parvovirus* επιλέχθηκε ως παθογόνος παράγοντας με ιδιαιτέρη σημασία για τους σκύλους και άλλα άγρια είδη σαρκοφάγων ενώ η σαλμονέλλα παραμένει η δεύτερη συχνότερη αιτία γαστρεντερίτιδας σε ανθρώπους στην Ευρωπαϊκή Ένωση, δηλαδή αποτελεί μικροοργανισμό με μεγάλη σημασία για τη Δημόσια Υγεία.

6Στο πρώτο κεφάλαιο της μελέτης, έγινε κριτική ανασκόπηση της διεθνούς βιβλιογραφίας και παρουσιάστηκαν κίνδυνοι για την ανθρώπινη υγεία που προκύπτουν από την αλληλεπίδραση ανθρώπων και ζώων. Πιο συγκεκριμένα, ζωονοτικοί παθογόνοι παράγοντες που εντοπίζονται στον εντερικό σωλήνα και μπορούν να μεταδοθούν μέσω των κοπράνων παρουσιάστηκαν σε κατηγοριοποίηση (Παράσιτα/ Πρωτόζωα/ Βακτήρια/ Ιοί). Μεταξύ αυτών, στελέχη των *Salmonellae*, *Campylobacter* spp., ροταϊών, *Toxoplasma gondii* και ο εχινόκοκκος αποτελούν πρόβλημα σε πολλές χώρες, συμπεριλαμβανομένης της Ελλάδας.

Η εκτίμηση της διαγνωστικής ακρίβειας μιας εμπορικής διαγνωστικής μεθόδου για την ανίχνευση του *Canine Parvovirus* περιγράφεται στο δεύτερο κεφάλαιο. Γίνεται σύγκριση της ταχείας εμπορικής δοκιμής με συγκεκριμένη PCR μέθοδο, που θεωρείται ως η πιο αξιόπιστη μέθοδος για την ανίχνευση του ιού. 78 δείγματα κοπράνων συλλέχθηκαν από σκύλους με διάρροια. Σε 12 σκύλους αναφέρθηκε εμβολιασμός σε διάστημα ενός μήνα πριν την έναρξη των συμπτωμάτων. Η ταχεία δοκιμή έγινε σε 23 δείγματα άμεσα, ενώ τα υπόλοιπα αποθηκεύτηκαν στους -20◦C. Η ευαισθησία της εμπορικής μεθόδου σε σχέση με την PCR ήταν 22.22% (95% CI: 13.27–33.57%) όσον αφορά το σύνολο των δειγμάτων, στα δείγματα από μη εμβολιασμένους σκύλους 26.67% (95% CI: 16.08–39.66%) και στα δείγματα που εξετάστηκαν αμέσως μετά τη συλλογή τους 76.47% (95% CI: 50.10–93.04%). Η ειδικότητα της δοκιμής ήταν 100%. Σύμφωνα με τα προαναφερθέντα αποτελέσματα, εξάγεται το συμπέρασμα ότι τα αρνητικά αποτελέσματα της ταχείας δοκιμής δεν αποκλείουν την ύπαρξη του ιού, αλλά το θετικό αποτέλεσμα σχεδόν σίγουρα υποδηλώνει μόλυνση από *CPV*. Η ταχεία δοκιμή είναι προτιμότερο να διεξάγεται, αμέσως μετά τη συλλογή των δειγμάτων.

Στο τρίτο κεφάλαιο, στόχος ήταν η ανίχνευση της παρουσίας του ιού *Canine Parvovirus* σε σκύλους με συμβατά κλινικά συμπτώματα στην Κεντρική Ελλάδα καθώς και η διαπίστωση ύπαρξης δημογραφικών και περιβαλλοντικών παραγόντων που ενδέχεται να παίζουν κάποιο ρόλο στην εμφάνιση του. Συνολικά εξετάστηκαν 117 δείγματα κοπράνων σκύλων έναντι τμήματος του γονιδίου *VP2*. Η λογιστική παλινδρόμηση, με τη χρήση του προγράμματος R, έδειξε ότι οι παράγοντες που σχετίζονται με την εμφάνιση του ιού είναι η ηλικία και η χρήση του σκύλου (οι νεαροί και οι σκύλοι εργασίας είναι πιο πιθανό να μολυνθούν από ότι οι σκύλοι αμιγώς συντροφιάς). Τα ευρήματα του Γεωγραφικού Πληροφοριακού Συστήματος σε συνδυασμό με το μοντέλο κατανομής ειδών ήταν τα εξής: (i) η πυκνότητα του κτηνοτροφικού κεφαλαίου, (ii) οι χρήσεις γης (συγκεκριμένα οι περιαστικές περιοχές) και η πυκνότητα ανθρώπινου πληθυσμού αναγνωρίζονται ως οι πιο σημαντικοί περιβαλλοντικοί παράγοντες για την εμφάνιση του ιού.

Το τελευταίο κεφάλαιο αφορά την ανίχνευση *Salmonella* spp., ενός σημαντικού ζωονοτικού παθογόνου, σε σκύλους και ευρωπαϊκούς καφετί λαγούς, ενός διαδεδομένου θηράματος. 50 δείγματα από θηρευμένους λαγούς και 117 δείγματα σκύλων εξετάστηκαν με αξιολογημένη μέθοδο PCR. Επιπλέον, έγινε χωρική ανάλυση με τη χρήση ΓΠΣ για τη διερεύνηση της πιθανής γεωγραφικής κατανομής και των σημαντικών περιβαλλοντικών παραγόντων για την παρουσία του βακτηρίου. Βρέθηκαν θετικοί 10 από τους λαγούς και 8 από τα 117 δείγματα σκύλων. Η ανάλυση ΓΠΣ σε συνδυασμό με το μοντελό κατανομής ειδών έδειξε τα εξής: για τους λαγούς σημαντικοί περιβαλλοντικοί παράγοντες ήταν η εποχικότητα της βροχόπτωσης, η βροχόπτωση κατά το πιο ψυχρό τρίμηνο του έτους και η πυκνότητα του κτηνοτροφικού κεφαλαίου. Αντιστοίχως, για σκύλους θετικούς στη σαλμονέλλα, ως σημαντικοί περιβαλλοντικού παράγοντες βρέθηκαν: η μέγιστη θερμοκρασία του πιο θερμού μήνα, η μέση θερμοκρασία του πιο θερμού μήνα, η πυκνότητα του κτηνοτροφικού κεφαλαίου και ο δείκτης βλάστησης για τον Αύγουστο. Η πυκνότητα του κτηνοτροφικού κεφαλαίου είναι ένας κοινός παράγοντας που σχετίζεται με την παρουσία σαλμονέλλας και στα δύο ζωικά είδη, υποδεικνύοντας ότι ενδεχομένως υπάρχει μετάδοση του παθογόνου μεταξύ των παραγωγικών ζώων, των σκύλων και της άγριας πανίδας. Αυτή η μελέτη, που έγινε για πρώτη φορά στην Ελλάδα υποδηλώνει ότι ο ρόλος των λαγών και των σκύλων στην απέκκριση και μετάδοση των *Salmonellae* χρειάζεται περαιτέρω διερεύνηση.

Σε αυτή τη διδακτορική διατριβή, έγινε εκτίμηση μιας εμπορικά διαθέσιμης διαγνωστικής μεθόδου για την παρβοεντερίτιδα, η οποία είναι ακόμα παρούσα στην ηπειρωτική Ελλάδα. Εξερευνά επίσης τη σχέση μεταξύ της παρουσίας του ιου CPV με συγκεκριμένους δημογραφικούς και περιβαλλοντικούς παράγοντες. Επίσης, η παρουσία της σαλμονέλλας ανιχνεύεται σε σκύλους με διάρροια και λαγούς, ενώ συνδέεται με περιβαλλοντικούς παράγοντες, οι οποίοι διαφοροποιούνται μεταξύ των δύο ζωικών ειδών, υποδεικνύοντας ότι σε αυτή την συγκεκριμένη περίπτωση αντανακλώνται οι συνήθειες των ξενιστών. Η συνεχής επιτήρηση των πληθυσμών σκύλων σε συνδυασμό με στοχευμένους πληθυσμούς ειδών άγριας πανίδας είναι δυνατό να δώσουν πληροφορίες για παθογόνους παράγοντες (ειδικά ζωονοτικούς) που κυκλοφορούν αποτελώντας κίνδυνο για την άγρια πανίδα, τα κατοικίδια ζώα και τους ανθρώπους.