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Recent Advances in Canine Medicine

Edited by Carlos Eduardo Fonseca-Alves

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Aims and Scope of the Series

Paralleling similar advances in the medical field, astounding advances occurred in Veterinary Medicine and Science in recent decades. These advances have helped foster better support for animal health, more humane animal production, and a better understanding of the physiology of endangered species to improve the assisted reproductive technologies or the pathogenesis of certain diseases, where animals can be used as models for human diseases (like cancer, degenerative diseases or fertility), and even as a guarantee of public health. Bridging Human, Animal, and Environmental health, the holistic and integrative "One Health" concept intimately associates the developments within those fields, projecting its advancements into practice. This book series aims to tackle various animal-related medicine and sciences fields, providing thematic volumes consisting of high-quality significant research directed to researchers and postgraduates. It aims to give us a glimpse into the new accomplishments in the Veterinary Medicine and Science field. By addressing hot topics in veterinary sciences, we aim to gather authoritative texts within each issue of this series, providing in-depth overviews and analysis for graduates, academics, and practitioners and foreseeing a deeper understanding of the subject. Forthcoming texts, written and edited by experienced researchers from both industry and academia, will also discuss scientific challenges faced today in Veterinary Medicine and Science. In brief, we hope that books in this series will provide accessible references for those interested or working in this field and encourage learning in a range of different topics.

Meet the Series Editor

Rita Payan Carreira earned her Veterinary Degree from the Faculty of Veterinary Medicine in Lisbon, Portugal, in 1985. She obtained her Ph.D. in Veterinary Sciences from the University of Trás-os-Montes e Alto Douro, Portugal. After almost 32 years of teaching at the University of Trás-os-Montes and Alto Douro, she recently moved to the University of Évora, Department of Veterinary Medicine, where she teaches in the field of Animal Reproduction and Clinics. Her primary research areas include the molecular markers of the endometrial cycle and the embryo–maternal interaction, including oxidative stress and the reproductive physiology and disorders of sexual development, besides the molecular determinants of male and female fertility. She often supervises students preparing their master's or doctoral theses. She is also a frequent referee for various journals.

Meet the Volume Editor

Carlos Eduardo Fonseca-Alves is a Full Professor of Internal Medicine in Small Animals at Paulista University – UNIP, Brazil. He obtained his DVM from the Federal University of Goias, Brazil, and completed a residency program on small animal internal medicine at UPIS University, Brazil. He earned his MSc and Ph.D. at São Paulo State University (UNESP), Brazil, in 2013 and 2016, respectively. Dr. Fonseca-Alves undertook a postdoctoral research fellowship at UNESP in 2016–2019 and at the University of Southern Denmark in 2019. He has experience in veterinary medicine, focusing on internal medicine and molecular and comparative oncology.

Contents

Preface

The history of dogs and humans dates back thousands of years. It is believed that dogs were the first species domesticated by humans, possibly as early as 15,000 years ago. In the beginning, dogs were used as working animals for different activities, including hunting, herding, and guarding livestock. Over time, dogs evolved into companions and became valued members of human families. In the modern era, dogs continue to serve as companions, working dogs, and service animals and have become an integral part of human society. Advances in veterinary medicine and the study of animal behavior have led to a deeper understanding of the relationship between dogs and humans. Among the advances, it is important to highlight improvements in dog cancer treatments, such as targeted therapies and immunotherapy, and advances in surgical techniques, including minimally invasive procedures. The development of new diagnostic tools, such as genetic testing and imaging technologies, and an increased understanding of the gut microbiome and its role in canine health are some of the most recent advances in canine health care. Advances in preventive care, including personalized vaccine protocols and nutrigenomics, have also gained importance.

For all these reasons, new studies with advances related to canine medicine are essential for improving dog care. This book discusses recent advances in canine medicine with a collection of chapters focused on different areas of canine medicine, including, infectious and degenerative diseases, immunology, and cancer. I would like to thank all the authors for their excellent contributions. I am also grateful to the staff at IntechOpen for all their support. Thank you so much.

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Section 1

Canine Infectious Disease

Chapter 1

Parvovirus Vectors: The Future of Gene Therapy

Megha Gupta

Abstract

The unique diversity of parvoviral vectors with innate antioncogenic properties, autonomous replication, ease of recombinant vector production and stable transgene expression in target cells makes them an attractive choice as viral vectors for gene therapy protocols. Amongst various parvoviruses that have been identified so far, recombinant vectors originating from adeno-associated virus, minute virus of mice (MVM), LuIII and parvovirus H1 have shown promising results in many preclinical models of human diseases including cancer. The adeno-associated virus (AAV), a non-pathogenic human parvovirus, has gained attention as a potentially useful vector. The improved understanding of the metabolism of vector genomes and the mechanism of transduction by AAV vectors is leading to advancement in the development of more sophisticated AAV vectors. The in-depth studies of AAV vector biology is opening avenues for more robust design of AAV vectors that have potentially increased transduction efficiency, increased specificity in cellular targeting, and an increased payload capacity. This chapter gives an overview of the application of autonomous parvoviral vectors and AAV vectors, based on our current understanding of viral biology and the state of the platform.

Keywords: parvovirus, AAV, recombinant viral vectors, gene therapy, vector biology

1. Introduction

Parvoviruses are among the smallest of eukaryotic viruses. They are subdivided into three major groups namely densoviruses, autonomous parvoviruses (APV), and dependoviruses [1]. Whereas densoviruses infect only insects, APV and dependoviruses infect vertebrate animals. APV replicate in proliferating target cells without the need of helper viruses but dependoviruses require helper virus functions for replication. Vector development has focused on three rodent APVs that can infect human cells, namely, LuIII, MVM, and H1. Dependovirus is also known as Adeno-associated virus (AAV) because dependovirus cannot replicate and form viral capsids in its host cell without the cell being coinfected by a helper virus such as an adenovirus, a herpesvirus, or a vaccinia virus [2–5]. AAVs of humans and of numerous other vertebrates are known. More than 90% of human adults have antibodies to AAV, which shows that the virus is common and widely distributed. AAV serotypes 2, 3 and 5 are endemic in humans; AAV-4 infects mainly nonhuman primates and the host for

serotypes 1, 6, 7 and 8 is unclear [2, 6–12]. It is noteworthy that APVs and AAVs do not cause disease in humans. Even though human exposure to AAV and H1 may lead to mild and harmless viraemia, B19 (of the Erythrovirus genus) is the only virus of the Parvovirinae subfamily known to cause pathogenicity in humans [13, 14].

In past few decades, parvoviruses have progressed from a biologically interesting observation into a crucial driver in human gene therapy. Its potential has been displayed in various preclinical and clinical research studies all around the world. Their small size, simple genetic composition and structure, and the high degree of flexibility and amenability of genome and capsid to genetic engineering are some of the key characteristics of these viruses with respect to their development and use as recombinant gene therapy vectors for DNA delivery.

2. Adeno-associated virus (AAV)

Gene therapy protocols using recombinant viral vectors have proven potentially useful in molecular medicine. AAV is one of the most actively investigated gene therapy vehicles. It is a small (25 nm), non-enveloped virus composed by an icosahedral capsid that contains a single-stranded, 4.7-kb DNA genome. AAV genome is comprised of two genes rep and cap that are flanked by two palindromic inverted terminal repeats (ITR). Rep encodes for proteins associated with replication of the viral DNA, packaging of AAV genomes, and viral genome integration in the host DNA [15]. Cap encodes for the three proteins that form the capsid. In recombinant AAV vectors (rAAV), DNA sequences of interest between the AAV inverted terminal repeats (ITRs) are cloned, eliminating the entire coding sequence of the wt AAV genome. In the absence of Rep proteins, ITR-flanked transgenes encoded within rAAV can form circular concatemers that persist as episomes in the nucleus of transduced cells [16]. During AAV assembly, rep and cap genes are provided in trans together with the adenoviral helper proteins required for AAV genome replication and packaging [17, 18]. The most common method of rAAV production is by triple transfection of HEK293 cells with three plasmids: one containing the transgene expression cassette flanked by the viral ITRs, a second packaging plasmid expressing the rep and cap genes and a third plasmid encoding for adenoviral helper genes [17, 19].

To date, 13 different AAV serotypes and 108 isolates have been identified and classified [15, 20]. AAV2 was one of the first AAV serotypes identified and characterized, including the sequence of its genome. As a result of the detailed understanding of AAV2 biology, most rAAV vectors generated today utilize the AAV2 ITRs in their vector designs. The sequences placed between the ITRs will typically include a mammalian promoter, gene of interest, and a terminator. Subtle differences in binding preferences, encoded in capsid sequence differences, can influence cell-type transduction preferences of the various AAV variants [21–23]. For example, AAV9 has a preference for primary cell binding through galactose [24], AAV2 uses the fibroblast/hepatocyte growth factor receptor and the integrins αVb5 and α5b1; AAV6 utilizes the epidermal growth factor receptor; and AAV5 utilizes the platelet-derived growth factor receptor [25]. A deeper understanding of the AAV capsid properties has made the rational design of AAV vectors that display selective tissue/organ targeting possible, thus broadening the possible applications for AAV as a gene therapy vector. Pseudotyping of rAAV vectors is used to generate tropism-modified vectors. rAAV2 genomes can be packed into capsids derived from other AAV serotypes, thus narrowing or broadening the affinity of the new viral vector for specific cell types.

AAV has been shown to be safe and effective in preclinical and clinical settings. Due to their oncogenic and immunogenic properties [26, 27], retroviral and adenoviral vectors may be associated with certain complications, but AAV has not been proven to cause any such pathological symptoms. Additionally, AAV possesses many desirable features like its ability to transduce nondividing cells [28, 29], broad host range [30], and the ability of the wild-type (wt) AAV genome to integrate site specifically into chromosome 19 in human cells [31, 32]. Besides, wt AAV has also been shown to possess antioncogenic properties [33]. AAV can infect not only actively dividing cells, but also quiescent cells, which makes it particularly valuable for many cell populations where viral and non-viral vectors are not sensitive to gene delivery, such as retinal cells and neuronal cells. The natural ability of AAV to infect quiescent cells has contributed to many significant advances in gene therapy, such as Luxturna (Spark Therapeutics) approved by the FDA for the treatment of Leber's congenital amaurosis [34].

In the past 20 years, the relevance of AAV vector-based therapy in clinical transformation has continued to increase, and it currently accounts for 8.1% of global gene therapy clinical trials. There are currently 17 gene therapies approved by the US FDA, including the AAV vector voretigene neparvovec rzyl (VN), which was developed by Spark Therapeutics in 2017 under the trade name Luxturna [34]. VN contains an AAV2 that wraps the RPE6 gene, which is used to treat biallelic RPE65-related retinal dystrophy, a rare genetic disease that leads to impaired visual function, declines with age, and ultimately leads to blindness. The second AAV-based gene therapy approved by the FDA in 2019 is Onasemogene abeparvovec xioi (OA), developed by AveXis under the trade name Zolgensma. OA uses AAV9 expressing a functional SMN1 transgene to treat type I spinal muscular atrophy (SMA1) in children under 2 years of age [35].

Most AAV successfully used in preclinical and clinical research is limited to natural capsid serotypes. The existence of neutralizing antibodies against AAV is still an important obstacle to systemic delivery [36]. These neutralizing antibodies interfere with the entry of AAV into target cells, intracellular transport and unpacking in the nucleus, thereby preventing transduction. Epidemiological studies have shown that neutralizing antibodies with different seropositivity rates can be found in 30–60% of the population. The most popular of these neutralizing antibodies is against AAV2, followed by AAV1. Another problem of AAV-mediated gene therapy is the size limit of the genome (4.7 kbp), including ITRs, leaving only a ~4.5 kbp size space for the transferred gene. Engineered AAV can be designed through capsid modification, surface coupling and encapsulation to solve the limitations of natural AAV [37]. A common goal of AAV engineering is to avoid inactivation by neutralizing antibodies in the blood circulation after systemic administration. Another benefit of AAV engineering is to improve targeted delivery and activation by binding tissue-specific ligands to the capsid, surface coupling and encapsulating materials. Engineered AAV can also be used to overcome the limited genome size and combine multiple treatment modalities for multimodal therapy.

3. Autonomous parvovirus vectors

Autonomously replicating parvovirus (ARP) can replicate in proliferating cells without the need for a helper virus. This is one feature that makes the ARPs attractive for potential vector production. ARPs are found in many species; they do not require a helper virus for replication, but they do require proliferating cells

(S-phase functions) and, in some cases, tissue-specific factors [38]. Most vector work has been focused on autonomous parvoviruses that can infect human cells, namely, LuIII, MVM (minute virus of mice), and H1, which are members of the rodent group of APVs. Autonomous parvoviruses were first isolated from human tumor tissue and it was then observed that they possess an onco-suppressive potential, inhibiting the formation of spontaneous and chemically or virally induced tumors in vivo and in vitro [39–41]. Autonomous parvoviruses express preferentially in cancerous cells and possess oncolytic activity that has led to their implication in potential use as vectors for cancer gene therapy. Moreover, these viruses do not cause pathogenicity in adult animals and they seem to be associated with low or no immunogenicity. Additional features that make APVs interesting candidates for gene therapy are their episomal replication and high stability [42]. APV vectors have packaging capacity for foreign DNA of approximately 4.8 kb, a limit that probably cannot be exceeded by more than a few percent.

The genome of ARP comprises of two nonstructural proteins and viral capsid proteins. The non-structural proteins, NS-1 and NS-2 are highly conserved among the rodent parvoviruses that lead to cross-reactivity in serological assays utilizing whole virus antigen. The viral capsid proteins, VP-1 and VP-2 are specific to the virus and form the basis for serological differentiation. All currently proposed MVM and H-1 vectors retain the palindromes and NS1-coding sequences [42]. Other than its role in viral DNA replication, NS1 also possess the cytotoxic activity in tumor cells which should contribute to the destruction of tumor cells directly, through oncolysis, and indirectly through the induction of an immune response via the presentation of tumor-associated antigens by APCs to lymphocytes [43]. In addition, the late promoter P38 that regulates the expression of capsid proteins is transactivated by NS1. This promoter is used for the expression of transgenes to ensure that their expression occurs only in cells that also express NS1. VP proteins are generally expressed from P38, either on a helper plasmid or in packaging cells, thereby linking the expression of capsid proteins to the viral life cycle [43].

The major problems encountered with these vectors are their low titers and the generation of wild-type or replication-competent virus (RCV) through recombination with helper plasmids [42]. Over the time, advancements have been made to enhance the titers of recombinant virus and to reduce the contamination by RCV. Genetic engineering of vector has led to their enhanced production after transfection [44]. The reduction of homology between vector and helper sequences as well as integration of helper sequences into host cell genomes have greatly reduced the generation of RCV [45, 46].

Similarly, production of LuIII transducing virus has been accomplished by co-transfection of plasmid-based helper and transducing genome constructs [47]. In general, during co-transfections to generate transducing virus, recombination between helper and transducing genomes can regenerate infectious virus with variable frequencies [47]. Elimination of DNA-DNA recombination can be achieved by providing one of the components of the packaging system in RNA form. Sindbis, the plus-strand RNA virus that can express large amounts of protein from foreign genes in a variety of vertebrate cell types [48], were used for providing components of the LuIII packaging system in RNA form. Sindbis replicon vector was used to express NS1, the major non-structural protein of LuIII. Sindbis-expressed NS1 RNA and protein were readily detectable in cultured cells; this NS1 was able to mediate production of LuIII-luciferase transducing virus [48].

4. Use of parvovirus vectors in cancer gene therapy

Gene therapy is one of the most promising approaches for cancer treatment because it has the potential to provide tumor cell selectivity and/or protection of untransformed cells of the body. In order to transduce the gene of interest, either nonviral vectors or viral vectors are used. Nonviral vector strategies include naked plasmid DNA, liposome-DNA complexes, peptide-bound DNA and electroporation [49]. The most widely used viral vectors are retroviruses, adenoviruses and herpesviruses. The preliminary data looks very promising, but most vectors suffer from downsides that limit their utility for gene therapy. While nonviral vector systems have low transfection efficiencies, most of the viral systems have the problems of poor tumor targeting, immunogenicity and low transduction efficiencies. Certain parvoviruses are characterized by their oncotropism, oncosuppression and ability to mediate long-term gene expression. Together with their human apathogenicity, these characteristics make them very interesting vector systems for cancer gene therapy. Viruses of the Parvovirinae subfamily, of the Parvoviridae family, have the ability to infect a variety of different vertebrates. Although the natural hosts of parvovirus H1, MVM and LuIII are rodents, these parvoviruses can also infect human cells [50]. Similarly, many AAV serotypes are endemic in humans and non-human primates. Despite this, neither of these viruses are pathogenic in humans.

Gene therapy strategies for tumor cells have to be highly specific, particularly when the vector is to be used systemically, in order to prevent damage to healthy tissues. The therapeutic index of most existing vectors is low [51]. They non-specifically transduce normal cells as well along with targeting the tumor cells resulting in undesired damage and cell death. In general, there are two different ways to achieve specificity of a gene therapy vector: transductional targeting and transcriptional targeting [51, 52].

Transductional targeting describes the selective uptake of the vector into the cells of interest, where the transgene is transcribed. Selective uptake can be achieved by various strategies, such as modification of the viral capsid or pseudotyping of viruses. AAVs have serotype-specific tissue tropism; thus, one approach to achieving tissue-specific transduction with a therapeutic gene is the use of different AAV serotypes [53, 54]. For example, AAV-2 preferentially transduces the liver, AAV-1 transduces the muscle and AAV-5 transduces airway epithelium [53, 55]. However, serotype-specific tissue tropism enhances transgene expression in certain tissues but does not provide absolute specificity of transgene expression in other tissues. Various re-targetting strategies have been tried to enhance the specificity, efficiency and safety of AAV vectors, in particular: (1) direct re-targeting by modification of the viral capsid using the optimal insertion site that ensures the presentation of a targeting peptide on the viral surface but does not interfere with packaging [56–58] and (2) indirect re-targeting using a molecule bound to the viral surface that binds specifically and stably to the target cell (e.g. glycoside molecules and bispecific antibodies) [59, 60]. In another study, two unique features of AAV and B19 virus were exploited to create a chimeric recombinant vector system to specifically target the primitive erythroid progenitors in human bone marrow cells [61]. Recombinant B19 virus vectors are much more efficient than the recombinant AAV vectors in transducing primary human erythroid progenitor cells. Further refinement of this vector system can be useful in cancer gene therapy applications for erythroid cell lineage in the human hematopoietic system.

In transcriptional targeting, even though the transgene might be taken up by many different cells, it is transcribed only in the target cells. Using this approach, transgenes are expressed selectively by replacing the natural promoter or by modifying the transcription-factor-binding sites within a promoter. Transcriptional targeting of AAV is mostly used to enhance transgene expression in a tissue-specific manner rather than to restrict its expression to certain tissues. There are various promoters that have been used successfully like an albumin gene promoter and a retroviral long terminal repeat promoter to express human α1 -antitrypsin in hepatocytes [62], a myelin basic protein (MBP) gene promoter to direct MBP expression specifically to oligodendrocytes [63, 64] and regulatory elements of the F4/80-gene promoter for specific expression in primary microglia [65]. Transcriptional targeting of ARPs has the benefit that the vectors are already selective for cancer cells. Transcriptional targeting of ARPs has been used to achieve cell-type specific transgene expression of the parvovirus LuIII. rLuIII vectors expressing the luciferase marker gene under the control of a chimeric promoter containing a liver-specific enhancer. It directed the preferential expression of the luciferase marker in transduced human hepatoma cells [66, 67]. Another approach targeted colon carcinoma by using hybrid H-1-MVM parvovirus vectors carrying binding sites for the heterodimeric β-catenin/Tcf transcription factor in the P4 promoter; this transcription factor functions in the wnt signalling pathway, which is constitutively activated in colon carcinoma.

Gene therapy strategies for cancer can be grouped as follows: (1) Immunogene therapy with the aim of achieving either an antitumor vaccine effect or enhancing T-cell antitumor effector capability; (2) anti-angiogenic gene therapy to reduce the supply of oxygen and nutrients to the tumor; (3) cytoreductive gene therapy by gene transfer to a large number of tumor cells in situ to achieve nonimmune tumor reduction by direct cytotoxicity or by an indirect bystander effect; and (4) transduction of HSCs with drug-resistance genes to enhance their resistance to cytotoxic drugs. Depending on the desired gene therapy approach, there are different requirements the vector must fulfil with regard to safety and efficiency of the vector, specific targeting of gene transduction, expression level of the transduced gene and ease of manufacture [68].

5. Conclusion

It has become increasingly clear that parvovirus-based vectors are a potentially safe and useful alternative to the more commonly used retroviral and adenoviral vectors. Gene transfer vectors based on the replication-defective (adeno-associated virus) and autonomous parvoviruses are emerging as promising vehicles for gene therapeutic approaches. AAV has been exploited as a gene delivery vector due to its unique characteristics like small size, simple genetic composition, lack of inflammatory response, and ability to transduce both dividing and non-dividing cells followed by persistence for the lifetime of the cell. AAV-based vectors are nonpathogenic and possess an extremely wide host and tissue range. Unlike AAV, autonomous parvoviruses do not integrate. However, their tropism for transformed tissues and innate oncolytic properties may permit rapid in situ therapies. As a consequence, APVs, including MVM and H-1 virus, have been developed as antitumor vectors with the aim of strengthening the antineoplastic effect of the natural parvoviruses. With the emergence of clinically approved products in the global market and more and more successful clinical trials being conducted, AAV is at the forefront of gene therapy, but

its smaller genome and neutralizing antibodies limit its application in many diseases. Present research suggests that the genetic modification of AAV vectors may further increase the success of AAV gene therapy. Vector can be engineered to increase AAV transduction efficiency by optimizing the transgene cassette. Moreover, capsid engineering can enhance vector tropism and the ability of the capsid and transgene to avoid the host immune response. Genetic manipulation of these components to optimize the large-scale production of AAV is also being explored.

APVs can prove to be superior alternative to more established vectors for gene transfer, particularly with respect to their potential use in cancer therapy. Based on the natural diversity of APVs and the ability to generate pseudo types with capsids from closely related members of the group, they should complement AAV vectors as well as offer various advantages like circumventing immune responses and exploiting tissue tropisms. However, substantial work is required to completely explore the pros and cons of these vectors, especially in context of mechanisms of transduction and the range of tissues that can be transduced in vivo by vectors with alternative, or modified, capsids. A significant preclinical evaluation of these vectors should lead to their application in future clinical cancer gene therapy trials.

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Chapter 2

The Diversity of Parvovirus Telomeres

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Abstract

Parvoviridae are small viruses composed of a 4–6 kb linear single-stranded DNA protected by an icosahedral capsid. The viral genes coding non-structural (NS), capsid, and accessory proteins are flanked by intriguing sequences, namely the telomeres. Telomeres are essential for parvovirus genome replication, encapsidation, and integration. Similar (homotelomeric) or different (heterotelomeric) at the two ends, they all contain imperfect palindromes that fold into hairpin structures. Up to 550 nucleotides in length, they harbor a wide variety of motifs and structures known to be recognized by host cell factors. Our study aims to comprehensively analyze parvovirus ends to better understand the role of these particular sequences in the virus life cycle. Forty *Parvoviridae* terminal repeats (TR) were publicly available in databases. The folding and specific DNA secondary structures, such as G4 and triplex, were systematically analyzed. A principal component analysis was carried out from the prediction data to determine variables signing parvovirus groups. A special focus will be put on adeno-associated virus (AAV) inverted terminal repeats (ITR), a member of the genus *Dependoparvovirus* used as vectors for gene therapy. This chapter highlights the diversity of the *Parvoviridae* telomeres regarding shape and secondary structures, providing information that could be relevant for virus-host interactions studies.

Keywords: parvovirus, telomeres, DNA folding, DNA secondary structure, adeno-associated virus

1. Introduction

Many linear DNA viruses possess terminal repeats (TRs) known to be critical for viral genome stability and propagation [1]. A parallel can be drawn with human chromosome telomeres that are composed of GC-rich repeat sequences of 5–10 nucleotides. In cells, telomeres are critical to maintain the linear structure of the chromosomes. They can adopt specific secondary structures, such as G-quadruplexes (G4), providing structural characteristics for protein binding and genomic stability. In addition, Cellular telomeres play a role in transcription regulation, chromatin compaction, subcellular localization, and chromosome segregation.

Similarly, *Parvoviridae* TRs have been demonstrated to be essential to several steps of the virus cycle. They vary in shape and size from approximatively 100 to 550

nucleotides [2]. Due to the presence of palindromic repeats, TRs can fold into T-, I-, J-, Y-, U- shape or simple hairpin-like structures. Up to now, the global shape has been named without any consensus, for example, Y-shape also being called "rabbit ears."

Viral genomes in some genera (*Ambi-* and *Itera-densovirus*; *Ave-*, *Dependo-*and *Erythro-parvovirus*) are homotelomeric meaning that both termini are similar but inverted, whereas, in other genera (*Brevi-* and *Hepan-densovirus*; *Amdo-*, *Boca-* and *Proto-parvovirus*), the 5′ and 3′ ends of the linear genome differ and therefore are called heterotelomeric. The strand polarity packed in viral capsids may be related to the left and right TRs dissimilarity. Indeed, most of the heterotelomeric parvoviruses encapsidate only one strand polarity, mainly negative. This preference may be due to inefficient nicking during replication or incomplete packaging signal at one TR [2]; for example, the minute virus of mice (MVM), a virus of the subfamily *Parvovirinae* and genus *Protoparvovirus*, harbors a Y-shape left end and a longer U-shape structure on its right end. After replication and ori resolution, the single-stranded DNA of minus polarity is preferentially displaced from the left TR and encapsidated. For parvovirus with both polarities, the proportion can range from 1 to 50% and may be influenced by the host cell in which the virus is produced [3].

Parvoviral TRs are involved in many steps of the virus life cycle. They contain most of the *cis*-acting information required for genome replication and encapsidation, including tetranucleotide repeats that serve as binding sites for NS1 (Rep) oligomer, a resolution site necessary for the completion of the DNA strand copy, and a packaging signal. Recognized as DNA double-strand breaks (DSB) in the host cell, TR can trigger a DNA damage response (DDR), leading to the circularization and concatemerization of the viral genomes either by non-homologous end-joining (NHEJ) or homologous recombination (HR) [4]. Finally, transcription regulation elements are contained in the genome ends. For example, the MVM TRs contain both symmetric and asymmetric binding sites for transcription factors that modulate expression from the adjacent P4 promoter [5] and the Acheta Domestica Densovirus TRs contain a TATA box used for transcription initiation of NS gene on one side and VP on the other side [6].

The TRs secondary structures, motifs composition, and their role in the virus-cell cycle have been under-examined. In this study, DNA secondary structures of the *Parvoviridae* TRs, including non-canonical secondary structures, have been predicted. We have shown a high diversity of parvovirus telomeres characteristics even within a genus. This chapter may provide significant knowledge for *Parvoviridae* classification and interaction with host cells.

2. The intriguing shape diversity of parvovirus telomeres

2.1 Size, GC content, and shape of parvovirus ends

Although *Parvoviridae* genomes have been extensively studied, in particular for phylogenetic and evolutionary analyses, the sequence and characteristics of their telomeres are not clearly described. Therefore, we analyzed *Parvoviridae* TRs sequences publicly available in the NCBI GenBank database. *Parvoviridae* complete genomes were downloaded. At least one representative virus per genus was selected based on their notoriety and information available on the internal committee on the taxonomy of viruses (ICTV) website. TRs were annotated following GenBank annotation or information available in the literature (**Table S1**). TR sequences of homotelomeric genomes were then verified by aligning the 5′ and 3′ ends. Sequences

differing in length and showing no homology (with no common palindromic regions between 5′ and 3′ ends) were discarded from the data set. Finally, the presence of palindromic regions was verified by RNAfold (method described later) [7]. A total of 40 *Parvoviridae* 5′ and the 3′ TRs sequences were extracted for further analysis. Among those are 17 *Densovirinae*, 22 *Parvovirinae,* and 1 unclassified *Parvoviridae* telomeres.

First, the length of each TR was determined and listed in **Table S1**. Interestingly, TR length varies within a single genus (**Table 1**), for example, going from 122 nucleotides for the PcDV to 550 nucleotides for the GmDV in the same genus *Ambidensovirus*. Second, the percentage of GC was calculated for each parvovirus TR. **Figure 1** highlights the GC content diversity of TRs between parvoviruses. The minimum and maximum GC content was observed for the left TR of AalDV2 and AAV2 with 32.4% and 69.7%, respectively. Within the genus *Ambidensovirus*, the percentage of GC ranges from 35% to 58.5% with the lowest GC content being attributed to the 5′ TR of the PcDV (**Figure 1b**). Comparatively, telomeres of the human chromosomes contain 50–55% of G and C bases whereas the whole human genome contains 40.9% GC on average [8].

To visualize the general shape and the secondary structures of the viral TRs, the folding of each parvovirus TR was predicted by RNAfold program using parameters of the Turner model for single-stranded RNA and DNA and the Matthews model for double-stranded DNA [7]. Additionally, mFold program was used in the DNA mode to corroborate the predictions [9]. The most thermodynamically stable structures, or minimum free energy (MFE) structures, obtained on the RNAfold web server were used to propose a classification of the TR (**Figure 2**). Four groups were constituted according to the number of hairpin loops at their extremity and named H1 (previously named U- and I-shapes in the literature), H2 (corresponding to J-, Y-, T-shapes), H3, and H4 (**Figure 2**, **Table S1**). This classification based on the number of terminal hairpin loops after folding and on additional structural characteristics may be more informative and precise than the global shape. Moreover, this nomenclature is applicable to all parvovirus TR. Interestingly, TR sequences and shapes differ within a genus. For example, among *Ambidensovirus*, CpDV and DicDV 5′ TRs are both classified in the H1 group although they only share 43% of sequence homology. In the genus *Bocaparvovirus*, HBoV1 and BpV1 left ends are 62% homologous in sequence but form a terminal H1 and H2 shape, respectively. Phylogenetic and evolution analyses of *Parvoviridae* have been constructed on the basis of NS1 proteins homology. Telomeres have never been considered as a classification criterion.

Subfamily	Genus	5′ TR length	3′ TR length
Parvovirinae	*Bocaparvovirus*	[140–161]	[161–200]
	Dependoparvovirus	[141–455]	[141–455]
	Erythroparvovirus	[94–383]	[94–383]
Densovirinae	*Ambidensovirus*	[122–550]	[122–550]
	Brevidensovirus	[98–182]	[134–165]
	Iteradensovirus	[101–271]	[101–271]

Table 1.
Minimal and maximal length of five-prime and three-prime terminal repeats within genera of the Parvoviridae *family.*

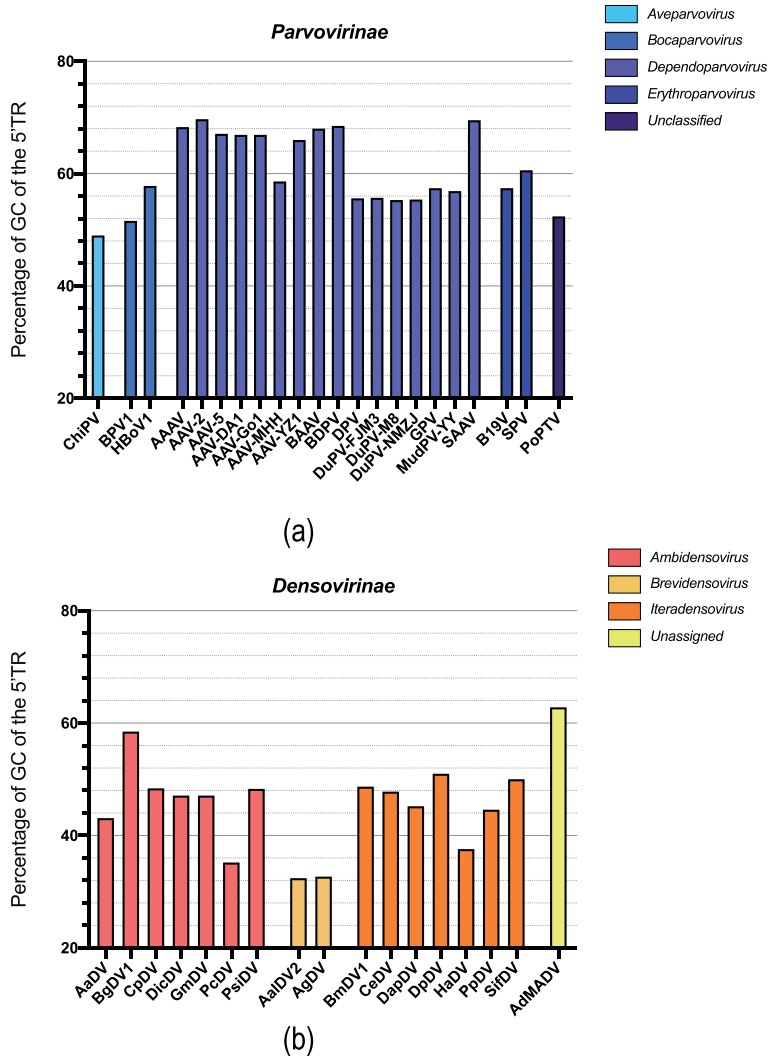


Figure 1.
GC content of 5-prime terminal repeats of the Parvoviridae *family: Differences inside the* Parvovirinae *(a) and the* Densovirinae *(b) subfamilies. The telomere sequences were downloaded from the NCBI GenBank database (see* ***Table S1*** *for accession numbers). GC content was calculated using the APE program.*

2.2 Comprehensive analysis of DNA secondary structural elements

The global analysis of the parvovirus TR has highlighted their broad diversity, even within the same genus. To study the TR divergence, an in-depth prediction of the secondary structures followed by a principal component analysis (PCA) have been realized. Secondary structure elements (**Figure 2**) and non-B form DNA structures were included as variables in the PCA.

Non-canonical specific structures are susceptible to be recognized by cellular proteins and thus to be essential in the virus-host interactions. For example, a recent study reported that special structures in DNA, such as quadruplex structures, can preferentially bind to IFI16 and trigger more potent type I IFN responses than those produced by the same sequence in dsDNA [10]. Such structures are intrinsic in many viral genomes,

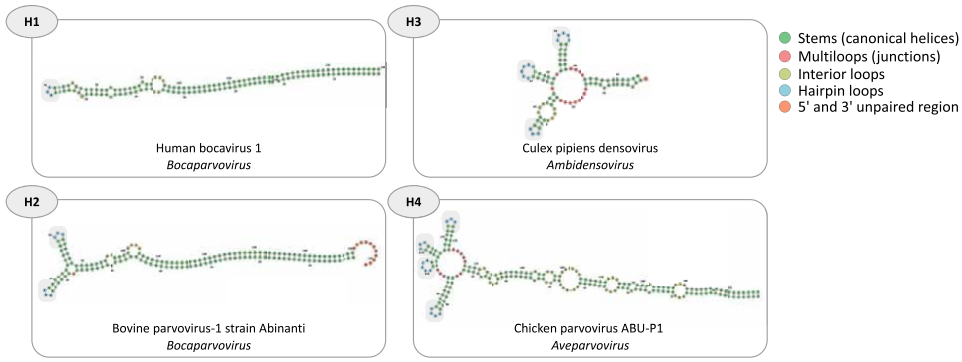


Figure 2.
Examples of five-prime terminal repeat folding among the Parvoviridae *family. The most thermodynamically stable structures or minimum free energy (MFE) structures were obtained using the RNAfold program (RNA mode: http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Four groups (H1 to H4) were generated according to the number of hairpin loops (in blue) found at the five-prime TR extremity. The following DNA secondary structures were identified and counted: Stems (green), multiloops (red), interior loops (yellow), and hairpin loops (blue).*

such as those of EBV and HPV [11]. Rich in GC, viral telomeres may also contain non-B DNA structures, such as G-quadruplexes (G4) or triplexes.

Therefore, putative G4 and triplexes were determined in all the parvoviral TR. G4 have been non-canonical DNA secondary structures formed by G-rich sequences (**Figure 3a**). Present in human telomeres, they are suggested to participate in chromosome stability maintenance [12]. G4 have also been shown to be present and play major roles in almost all virus families [13]. G4 have also been described in some parvovirus telomeres [14] but has not been systematically predicted in all parvovirus ends. G4 were predicted using the online tool QGRS-mapper [15] using the search parameters—QGRS max length 45, min G-group size 2, loop size from 0 to 12. These criteria are deliberately drastic to increase the stringency and relevance of the G4 prediction. Three values were collected—the raw number of predictive G4, with and without overlaps, and the QGRS max-score rewarding the G4 that are more likely to form. The *erythroparvovirus* B19V contains four G4 without overlaps which represent the maximum number of these non-B motifs for parvovirus TR. Including overlaps, CeDV and SifDV TRs harbor the highest number of putative tetraplex DNA structures with 296 G4. Of note, the two *brevidensoviruses* lack any predictive G4 in their ends. No correlation exist between the length of TR and the number of predictive G4 (data not shown).

In parallel, triplexes are important non-B form DNA structures for protein recognition, such as for the binding of p53 factor [16]. Triplex can form at homopurine: homopyrimidine sequences with mirror symmetry (**Figure 3b**). The triplex package of the R program was used to predict the existence of intramolecular triplex DNA structures in parvovirus TR [17]. Only two triplexes were found, both in the *bocaparvovirus* BAAV ends.

Finally, a PCA was performed for the forty left TRs and with the following variables—length, GC content, shape, max G-score, raw number of G4 with overlaps, and secondary structures elements (hairpins loops, interior loops, junction loops, and stems) collected from RNAfold analysis. The R package FactoMineR was used [18]. The main PCA variables are the stems and loops (**Figure 4a**). The "hairpin loops" criteria is one of the most important element allowing division of parvoviruses into groups, hence the relevance of our proposed classification in shapes H1 to H4. Clustering was subsequently realized on the three most informative dimensions

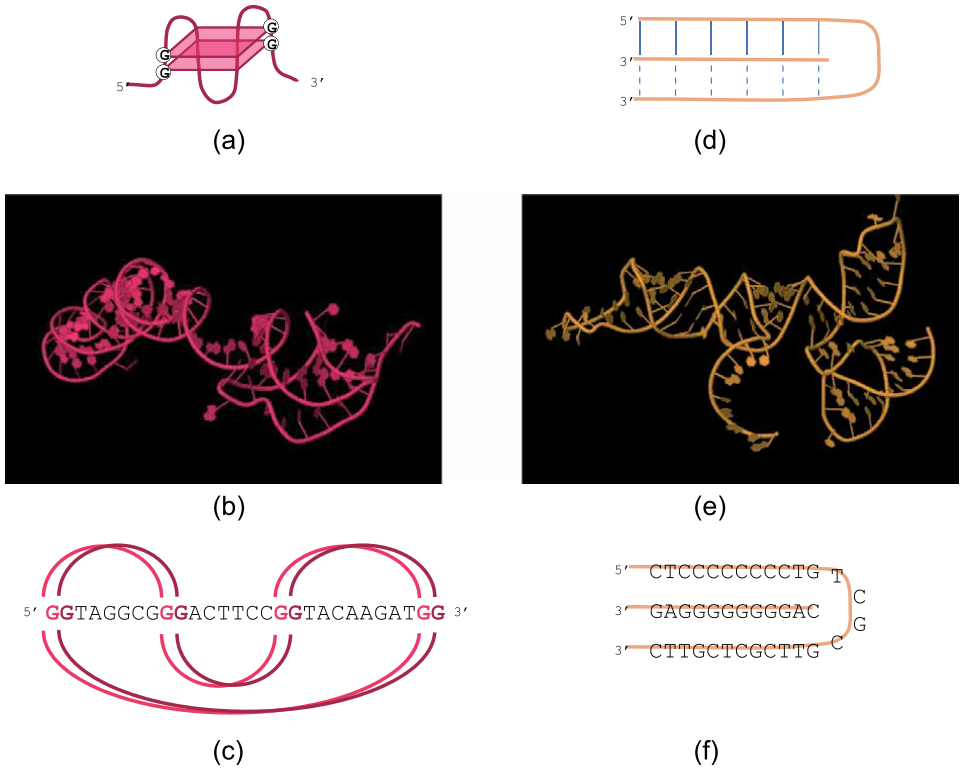


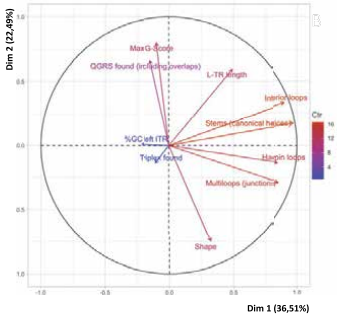
Figure 3.
Two non-B secondary structures: G-quadruplex (G4) and triplex. (a) 3D representation of a theoretical G4. (b) 3D representation of a G4 in the parvovirus B19 five-prime telomere and correspondence to the G tetrads sequence (c). (d) Triplex theoretical 2D representation. (e) 3D representation of a triplex of the five-prime bovine AAV terminal repeat and (f) correspondence to the sequence. (b) and (e) figures were obtained using the Jmol software (Jmol: An open-source Java viewer for chemical structures in 3D. http://www.jmol.org/).

corresponding to more than 70% of the cumulative variance. Five clusters were obtained (**Figure 4b**).

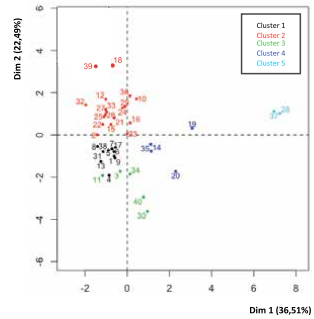
Cluster 1, composed of individuals such as the HBoV1 and AAV2, one of the most famous parvoviruses in the gene therapy field, is characterized by a high value for the variable "GC content." Parvovirus B19 belongs to cluster 2, a group characterized by high values for the G4 scores and TR length. Cluster 3 mainly depends on the shape class. Individuals in cluster 4 hold a similar number of multiloops and hairpin loops. Finally, viruses in cluster 5 share many DNA structure common features (stems, interior loops, hairpin loops, multiloops, and length). Clusters do not perfectly correlate with phylogenetic classification (**Figure 4c**), however, we observed that cluster 1 is only composed of *Dependoparvovirus* and *Bocaparvovirus* and cluster 5 contains two *Ambidensovirus*, GmDV and PsiDV. Interestingly, the latter highly differs from other groups.

3. Focus on adeno-associated virus (AAV) inverted terminal repeats (ITR)

The use of vectors derived from the adeno-associated virus (AAV) for gene delivery encounters a growing success for the treatment of a variety of human diseases



(a)



(b)

	Abbrevation	Genus	Cluster
1	AAAV	*Dependoparvovirus*	1
4	AAV-2	*Dependoparvovirus*	1
5	AAV-5	*Dependoparvovirus*	1
6	AAV-DA1	*Dependoparvovirus*	1
7	AAV-Go1	*Dependoparvovirus*	1
8	AAV-MHH	*Dependoparvovirus*	1
9	AAV-YZ1	*Dependoparvovirus*	1
13	BAAV	*Dependoparvovirus*	1
17	BPV1	*Bocaparvovirus*	1
31	HBoV1	*Bocaparvovirus*	1
38	SAAV	*Dependoparvovirus*	1

	Abbrevation	Genus	Cluster
2	AaDV	*Ambidensovirus*	2
10	AdMADV	*Unassigned*	2
12	B19V	*Erythroparvovirus*	2
15	BgDV1	*Ambidensovirus*	2
16	BmDV1	*Iteradensovirus*	2
18	CeDV	*Iteradensovirus*	2
21	DapDV	*Iteradensovirus*	2
22	DicDV	*Ambidensovirus*	2
23	DpDV	*Iteradensovirus*	2
24	DPV	*Dependoparvovirus*	2
25	DuPV-FJM3	*Dependoparvovirus*	2
26	DuPV-M8	*Dependoparvovirus*	2
27	DuPV-NMZJ	*Dependoparvovirus*	2
29	GPV	*Dependoparvovirus*	2
32	MokPV	*Unclassified Parvoviridae*	2
33	MudPV-YY	*Dependoparvovirus*	2
36	PpDV	*Iteradensovirus*	2
39	SifDV	*Iteradensovirus*	2

	Abbrevation	Genus	Cluster
3	AalDV2	*Brevidensovirus*	3
11	AgDV	*Brevidensovirus*	3
30	HaDV	*Iteradensovirus*	3
34	PcDV	*Ambidensovirus*	3
40	SPV	*Erythroparvovirus*	3
14	BDPV	*Dependoparvovirus*	4
19	CpDV	*Ambidensovirus*	4
20	ChiPV	*Aveparvovirus*	4
35	PoPTV	*Unclassified Parvovirinae*	4
28	GmDV	*Ambidensovirus*	5
37	PsiDV	*Ambidensovirus*	5

(c)

Figure 4.
Principal component analysis (PCA) conducted on the five-prime telomere repeats data set. The PCA analysis was conducted using the R software and the Factoshiny package. (a) Contribution graph of each variable. (b) Clusterisation of the 40 parvovirus TR. (c) Correspondence of each parvovirus in the 5 clusters.

[19]. Nevertheless, the scientific community has recently faced tragic toxicity of AAV vectors administered intravenously at high doses in several clinical trials [20]. AAV vectors are generated by inserting a recombinant genome usually flanked by AAV-2 inverted terminal repeats (ITR) in an AAV capsid. The recent side-effects observed in human trials have raised the question of DNA sensing, in particular, ITR detection and subsequent cellular responses [21]. Considering the importance of providing new knowledge in this field, a special focus on AAV-2 ITR was included in our study.

The homotelomeric AAV2 possesses two identical ITR of 145 nucleotide-long. The first 125 bases contain three palindromic sequences allowing the ITR to form a T-shape structure composed of two small inverted repeat sequences (BB′ and CC′) and a larger repeated sequence (AA′) (**Figure 5b**). According to our analysis, AAV2 ITR belongs to group H2 (**Figure 5c**). A fourth proximal region called D remains single-stranded if not annealed to the opposite polarity strand or not in an intramolecular manner to the D′ region in 3′. Each ITR can be found in two alternative configurations termed "flip" and "flop" distinguishable by the BB'-CC' orientation (**Figure 5a**), as a direct result of the replication mechanism. There are nomenclature inconsistencies in the literature. Here, ITR regions are named based on Lusby and Berns' publication [22] and ordered as followed ABB′CC′A′D from 5′ to 3′.

For historical reasons and the sake of convenience, most of the AAV vectors contain the ITR of AAV serotype 2, the sole viral sequences required for the

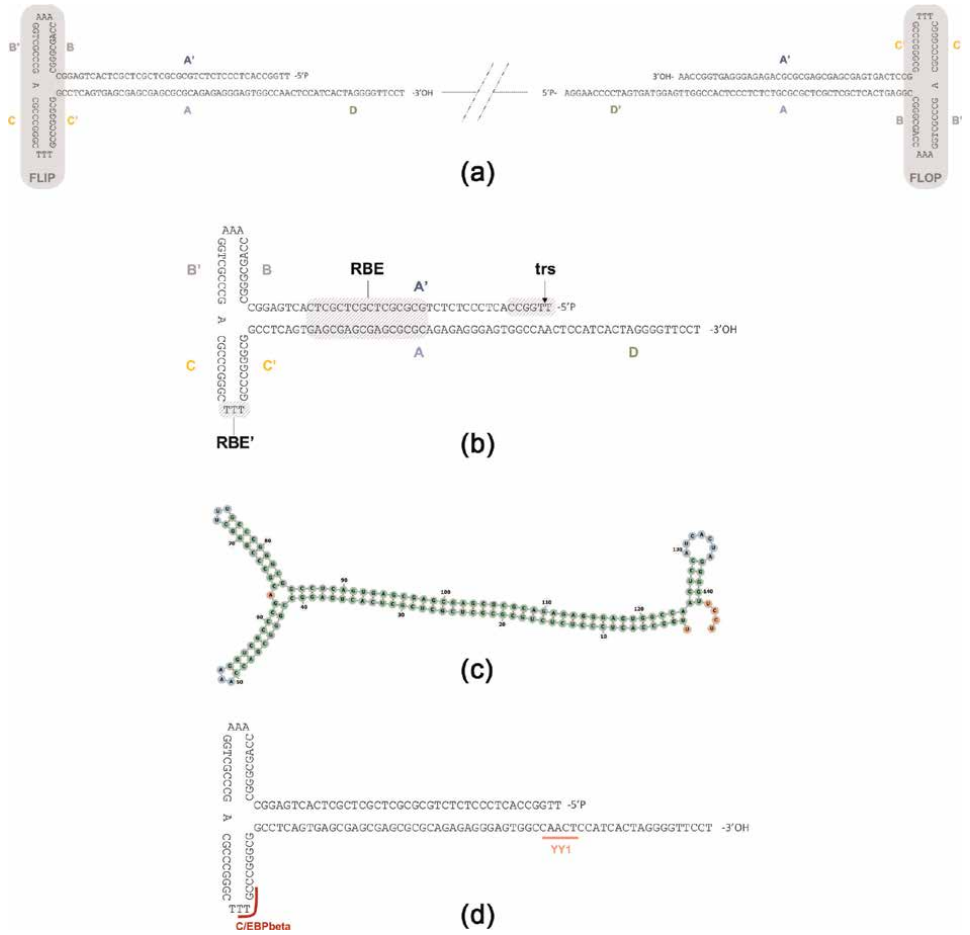


Figure 5.
Inverted terminal repeats of the adeno-associated virus (AAV) serotype 2. (a) Scheme of five prime and three-prime ITRs of the wild-type AAV serotype 2. (b) Two-dimensional drawing of the five-prime ITR in flip configuration. (c) Predictive folding of the five-prime AAV2 ITR using RNAfold. The color code is the same that for ***Figure 2*** *(http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). (d) Putative human transcription factor binding sites in AAV2 ITR. ITR regions are named based on Lusby and Berns' publication [22].*

replication and packaging of the recombinant genome in AAV capsids. Additional functions of AAV2 ITR have been described, such as a promoter activity [23], a role in the virus persistence either through genome integration [24] or recombination to form monomeric or concatemeric episomes [25].

Strikingly, the GC content of AAV2 ITR corresponds to the highest score of all studied parvoviral telomeres (69%). No predictive G4 was found using stringent parameters, unlike Satkunanathan *et al.* who described 18 QGRS inside the AAV2 ITR sequences [14]. In addition, no potential triplex structure was found. According to the PCA (**Figure 4**), AAV2 belongs to cluster 1 with several other *Dependoparvoviruses*.

Putative binding sites for human transcription factors (TF) have already been described in AAV2 ITR [26, 27]. We completed this work by analyzing human TF for AAV serotypes 1 to 7 ITRs using the Alggen-Promo tool with 0% sequence dissimilarity (**Table 2**) [28, 29]. Five human TF sites were found in AAV ITR: C/EBPbeta, Pax-5, YY1, AP-2alphaA, and GR-alpha. The C/EBPbeta was found in most of the

Serotype	Accession number	Transcription factors				
		C/EBPbeta [T00581]	Pax-5 [T00070]	YY1 [T00915]	AP-2alphaA [T00035]	GR-alpha [T00337]
1	NC_002077	2	2	1	1	0
2	NC_001401	1	0	1	0	0
3A	JB292182.1	1	2	2	0	0
3B	AF028705.1	1	2	1	0	0
4	NC_001829	1	0	1	0	0
5	NC_006152.1	2	0	0	0	2
6	AF028704	1	0	1	0	0
7	NC_006260	0	2	1	1	0
AAV_CHC1017	MK139265.1	1	0	1	0	0

Table 2.
Putative recognition sites of human transcription factors in inverted terminal repeats of AdenoAssociated virus serotypes. Predictions was realized using Alggen-promo tool with 0% sequence dissimilarity (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

AAV serotypes and unlike the other found TF, is mainly involved in immune responses. In our study, the GR-alpha was only found in AAV5 ITR contains two predictive TF binding sites, one for C/EBPbeta and other for YY1 (**Figure 5d**). YY1 participates in the initial steps of replication by binding to the p5 promoter region of AAV [30, 31].

4. Conclusion

The genomic and structural diversity of parvovirus is today classified by phylogeny analysis showing an expected separation between parvoviruses and densoviruses, but its robustness is relative, suggesting that the introduction of new sequences could change our perception of their evolutionary history [32]. The diversity of sequences, structures, and genomic organizations of parvoviruses suggest evolutionary histories that are probably more complex than those illustrated by current phylogenies. These observations led us to analyze and characterize the intriguing terminal sequences present in all parvoviruses, namely the telomeres.

This chapter highlights the diversity of *Parvoviridae* telomeres through a complete analysis of the terminal ends folding and secondary structures. Their length, GC content, and global shape vary even within a genus between phylogenetically closely related viruses. Evolution also led to heterotelomeric viruses with completely different left and right extremities. The diversity suggests high importance of these particular structures. Yet, factors involved in the TR selective pressure are unknown. Cotmore and Tattersall suggested a link between the resolution mechanism, the strand polarity, and the TR conformation [4, 33], while Tijssen et al. suggested that the significant differences in size and secondary structure of genome end between genera might reflect a dependence on specific cellular factors necessary for replication and encapsidation [34]. Consistently, we hypothesized that TR may have evolved according to the interactions with their replicase, helper virus co-factors, and/or cell

host proteins. Based on data integration of predictive DNA secondary structures in a PCA, new groups were made that were distinct from the ICTV phylogenetic classification conducted from the NS replicase sequences.

Additionally, the significance of specific secondary structures in the parvovirus life cycle and the relation with strand polarity of the packaged linear genome are interesting topics deserving further investigations. The MVM, canine parvovirus (CPV), BPV1 (*Parvovirinae*), and the AalDV2 (*Densovirinae*) encapsidate only or predominantly negative-strand polarity genomes and possess heterotelomeric TR [35, 36]. On the contrary, the homotelomeric AAV2 encapsidates both strands polarities at the same level. By having different shapes and different secondary structure elements, the TR directly impacts the polarity of the encapsidated strand.

Finally, a special emphasis was put on the ITRs of the adeno-associated virus serotype 2, taking into consideration its importance in the world of gene transfer using viral vectors. Particular motifs and secondary structures within AAV ITR may have a significant impact on gene transfer efficiency. Indeed, it has already been demonstrated that AAV2 ITRs are detected by cellular factors belonging to the NHEJ and HR-DNA damage pathways [37]. The viral telomeres may also be recognized by DNA sensors which subsequently could restrict AAV vectors transduction or activate innate immune responses [21]. Consistent with this hypothesis, a variety of cellular proteins have been shown to interact with AAV2 ITR, such as nucleophosmin (NPM1), a protein involved in ribosome biogenesis and nucleolus transport of basic proteins. Notably, NPM1 binds preferentially G4. The restriction factor FKBP52 in its phosphorylated form also binds to the ITR in the D region, inhibits the second strand synthesis, and consequently decreases transgene expression [38]. Thus, the involvement of ITR recognition by cellular factors is central to understand the extent of subsequent responses to the rAAV DNA that can negatively impact the therapeutic gene expression and cause potential safety concerns for the patients. Using drastic parameters, no putative G4 or triplex were found in AAV2 ITR contrary to a previous study [14]. The formation of these non-conventional DNA motifs highly depends on the adjacent sequences as well as pH and ion concentration conditions and thus requires to be confirmed experimentally.

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Conflict of interest

The authors declare no conflict of interest.

Appendix

Subfamily	Genus	Virus name	Abbreviation	Accession number	5′ TR length	5′ TR shape	3′ TR length	3′ TR shape	Reference used for TR annotation
Parvovirinae	*Aveparvovirus*	Chicken parvovirus ABU-P1	ChiPV	GU214704.1	206	H4	206	H4	[39]
	Bocaparvovirus	Bovine parvovirus-1 strain Abinanti	BPV1 HBoV1	NC038895	161	H2	161	H1	[40]
		Human bocavirus 1		JQ923422	140	H1	200	H1	[41]
	Dependoparvovirus	adeno-associated virus 2	AAV-2	NC_001401	145	H2	145	H2	[42]
		adeno-associated virus 5	AAV-5	NC_006152.1	167	H2	167	H2	[43]
		Adeno-associated virus isolate MHH-05-2015	AAV-MHH	NC040671	174	H2	174	H2	[44]
		Adeno-associated virus-Go.1 (caprine)	AAV-Go1	DQ335246	167	H2	167	H2	[45]
		Avian adeno-associated virus ATCC VR-865	AAAV	NC004828	142	H2	142	H2	[46]
		Avian adeno-associated virus strain DA-1	AAV-DA1	NC006263	142	H2	143	H2	[47]
		Avian adeno-associated virus strain YZ-1	AAV-YZ1	GQ368252	141	H2	141	H2	[48]
		Bearded dragon parvovirus	BDPV	NC027429	257	H2	257	H2	[49]
		Bovine AAV	BAAV	NC005889	172	H2	172	H2	[50]
		Duck parvovirus strain FJM3	DuPV-FJM3	KR075690	359	H1	359	H1	[51]

Subfamily	Genus	Virus name	Abbreviation	Accession number	5′ TR length	5′ TR shape	3′ TR length	3′ TR shape	Reference used for TR annotation
		Duck parvovirus strain M8	DuPV-M8	KR029614	387	H1	387	H1	[52]
		Duck parvovirus strain NMZJD110	DuPV-NMZJ	KR075691.1	415	H1	415	H1	[52]
		Goose parvovirus	GPV	U25749	444	H1	444	H1	[52]
		Muscovy duck parvovirus FM	DPV	NC_006147.2	457	H1	455	H1	[51]
		Muscovy duck parvovirus YY	MudPV-YY	KU844281	452	H1	452	H1	[51]
		Serpentine adeno-associated virus	SAAV	NC006148	154	H2	154	H2	[53]
	Erythroparvovirus	B19 virus isolate J35 Simian adeno-associated virus	B19V	AY386330.1	383	H1	383	H1	[54]
			SPV	KT984498	94	H3	95	H3	[55]
	Unclassified Parvovirinae	Porcine partetravirus strain FMV10-1437266	PoPTV	NC022104.1	210	H2	210	H2	[56]
Densovirinae	*Ambidensovirus*	Acheta domestica densovirus	AaDV	HQ827781	144	H1	144	H1	[57]
		Blattella germanica densovirus 1	BgDV1	NC005041	217	H1	216	H1	[58]
		Culex pipiens densovirus	CpDV	NC012685	285	H1	285	Unclassified	[59]
		Diaphorina citri densovirus	DicDV	NC030296.1	210	H1	210	H1	[60]
		Galleria mellonella densovirus	GmDV	NC_004286	550	H2	550	H2	[61]
		Planococcus citri densovirus	PcDV	NC004289.1	122	H2	122	Unclassified	[62]
		Pseudoplusia includens densovirus	PsiDV	NC019492.1	540	H2	540	H2	[63]
	Brevidensovirus	Aedes albopictus densovirus 2	AalDV2	NC004285.	182	H2	134	H2	[64]
		Anopheles gambiae densonucleosis virus	AgDV	NC_011317.1	98	H2	165	H2	[65]

Subfamily	Genus	Virus name	Abbreviation	Accession number	5′ TR length	5′ TR shape	3′ TR length	3′ TR shape	Reference used for TR annotation
	Iteradensovirus	Bombyx mori densovirus 1	BmDV1	NC003346.1	230	H1	230	H1	[66]
		Casphalia extranea densovirus	CeDV	NC004288.1	230	H1	230	H1	[67]
		Danaus plexippus plexippus iteravirus isolate Granby	DapDV	NC023842	239	H1	239	H1	[68]
		Dendrolimus punctatus densovirus	DpDV	NC006555.1	200	H2	200	H2	[69]
		Helicoverpa armigera densovirus	HaDV	NC015718	101	H4	101	H1	[70]
		Papilio polyxenes densovirus	PpDV	NC018450.1	271	H1	271	H1	[71]
		Sibine fusca densovirus	SifDV	NC018399.1	230	H1	230	H1	[72]
	Unassigned	Acheta domesticus mini ambidensovirus isolate Kalamazoo	AdMADV	NC022564.1	199	H2	199	H2	[73]
Unclassified Parvoviridae		Mouse kidney parvovirus strain Centenary Institute	MokPV	NC040843.1	145	H1	118	H1	[74]

The phylogenetic classification used here refers to the most up-to-date from the International Comitee on Taxonomy of Virus (ICTV) published in 2020. Abbreviations were taken from the literature; when not existing, they were created taking the first letters of the virus name. The TR shape were annotated according to our classification proposed in this chapter. The reference used for ITR annotations does not always match with the first citation of the virus.

Table S1.
List of the forty Parvoviridae *terminal repeats analyzed in the study.*

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Chapter 3

Canine Parvovirus-2: An Emerging Threat to Young Pets

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Abstract

Canine parvovirus-2 (CPV-2) is a highly contagious and key enteropathogen affecting the canine population around the globe by causing canine parvoviral enteritis (CPVE) and vomition. CPVE is one of the the leading causes of morbidity and mortality in puppies and young dogs. Over the years, five distinct antigenic variants of CPV-2, namely CPV-2a, CPV-2b, new CPV-2a, new CPV-2b, and CPV-2c, have emerged throughout the world. CPV-2 infects a diverse range of wild animals, and the newer variants of CPV-2 have expanded their host range to include felines. Despite the availability of highly specific diagnostics and efficacious vaccines, CPV-2 outbreaks have been reported globally due to the emergence of newer antigenic variants, expansion of the viral host range, and vaccination failures. The present chapter describes the latest information pertaining to virus properties and replication, disease manifestations in animals, and an additional recent updates on diagnostic, prevention and control strategies of CPV-2.

Keywords: Canine parvovirus-2, CPVE, myocarditis, young dogs and antigenic variants

1. Introduction

Canine parvovirus (CPV-2) is a member of the *Parvoviridae* family, *Parvovirinae* subfamily, and *Protoparvovirus* genus. It causes severe, acute hemorrhagic gastroenteritis and myocarditis infection in dogs [1]. It is the most important enteric virus affecting domestic and wild carnivores throughout the globe [2]. CPV-2 is a non-enveloped virus with a single-stranded negative-sense DNA genome [3]. The genetic diversity of CPV-2 resulted in the emergence of 5 distinct antigenic variants such as CPV-2a, CPV-2b, new CPV-2a, new CPV-2b, and CPV-2c with amino acid differences mainly restricted to the capsid VP2 protein [4]. CPV-2 are ubiquitous and sturdy viruses that remain viable for more than one year in the favorable environment [5, 6] and are transmitted usually by the faeco-oral route [7, 8].

CPV-2 causes 100 percent morbidity and mortality rate of 10 percent and 91 percent in adult and young dogs respectively [9]. However, a mortality of 91 percent was reported in experimentally infected dogs that were not treated [10]. CPV-2 affects predominately the younger dogs between 6 weeks and 6 months [8] with an increased

susceptibility to puppies less than 6 months. In dogs over the age of 6 months, sexually intact males are more likely (twice) to develop canine parvovirus enteritis (CPVE) in comparison to intact females [11]. The CPV-2 antibody titer transmitted to the newborn via absorbed colostral antibody is 50–60% of the mother's titer. The half-life of paroviral maternal antibodies is around 10 days [12]. Therefore, puppies are highly susceptible to the CPV-2 infection as the maternal antibody titres start declining. CPVE affects dogs of all ages, although it is more severe in puppies. Puppies can succumb to shock and die within two days after being sick. The most striking symptom of CPV-2 myocarditis is the abrupt mortality in young puppies, generally around the age of 4 weeks [13].

In recent years, CPVE outbreaks caused by multiple CPV-2 variants have been recorded in diverse geographical locations throughout the world. Previously, CPV-2, which could not infect cats, has been replaced by CPV-2 variants that can now infect cats, suggesting that CPV-2 may be capable of spreading between species [14]. Since CPV-2 infects a wide range of wild animals in the order Carnivora, subclinical infection appears to be prevalent. As a result, significant CPV-2 reservoirs in wildlife appear to exist, and transmission of virus between domestic dogs and wildlife appears to be common and bidirectional [15]. Despite the availability of a wide range of immunoprophylactic and antiviral agents to control CPV-2 infections in dogs, many outbreaks have been reported throughout the world, and the disease has remained a major veterinary and economic concern due to the presence of unvaccinated dogs, intervention of active immunization by maternally derived antibodies, and the emergence of a different antigenic variants of CPV-2.

2. Virology of CPV-2

Canine parvovirus infection is caused by *Carnivore protoparvovirus-1* which is characterized under the genus *Protoparvovirus*, family *Parvoviridae*. CPV-2 is a member of the *Parvoviridae* family, which includes two subfamilies: *Parvovirinae* (infects vertebrates) and *Densovirinae (infects invertebrates) Parvovirus, Erythrovirus, Dependovirus, Amdovirus, Bocavirus,* and other unclassified vertebrate parvoviruses are all the genus which comes under *Parvovirinae* (**Figure 1**) [16]. CPV-2 genome is a single stranded, negative sense, linear DNA of about 5 kb [17] contained by two ORFs translated into 4 proteins through alternative splicing [18, 19]. One ORF is associated with the non-structural proteins NS1 and NS2, which are mainly related to the viral replication and

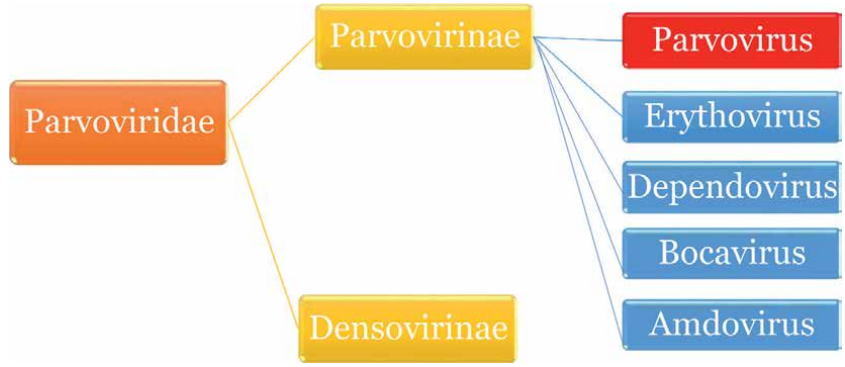

Figure 1.
Schematic representation of Parvovirdae *taxonomy.*

the second ORF is related with the viral capsid constituents VP1 and VP2. After the cleavage of VP2, VP3 is formed due to the involvement of host proteases. The capsid has 60 protein subunits, 90% of which are VP2 (67 kDa) and 10% are VP1 (83 kDa) [20].

The virus is nonenveloped having icosahedral symmetry and is 25 nm in diameter. The CPV virus is made up of the sixty protein subunits containing VP1 (5–6 units) and VP2 (54–55 units). The protein structure is made up of antiparallel β-barrel (8-stranded) capsid. The viral replication occurs inside the nucleus of multiplying cells and therefore the intranuclear inclusion bodies are formed during the infection. The viral capsid structure is made up of spike at the three-fold axes of the icosahedral unit, a 15-Å depression around the five-fold axes and two-fold axes is formed. Antigenic determinant regions have been plotted to the three-fold protrusion and the two-fold depression are related to the host cell features [17]. The surface of the capsid is composed of four loops inserted between the strands, resulting in spike-like protrusions around threefold axes of approximately 22 Å. The antigen neutralization site, also known as epitope A, is composed of loops 1 and 2 of one VP2 and loop 4 of a threefold related molecule [21]. The molecular weight (MW) is around 5.5 to 6.2×10^6 Da. There is an equal ratio of protein to nucleic acid.

NS1 is the largest non structural protein in CPV-2, and it is primarily involved in viral replication and pathogenicity [22]. NS1 is a key mediator of cytotoxicity of CPV and can selectively cause tumor cell lysis by inducing an antitumor immune response in different tumor models [23]. A recent study demonstrated the amino acid residues of T598 and T601 in the C-terminal phosphorylation sites of NS1 protein, involved in replication and pathogenicity of CPV-2 [24].

In the 1970s, CPV-2 emerged as a novel pathogen in dogs. Since then, CPVE has been reported across all the continents [25, 26]. Other related viruses such as Feline panleukopenia virus (FPV), Mink enteritis virus (MEV), Raccoon parvovirus (RPV) are closely related to the CPV-2 [27]. Mutations in the canine transferrin receptor (TfR) type-1 lead to adaptation of CPV-2 in different species 2 [28, 29]. There is more than 98% genome homology reported in the CPV and FPV nonetheless infect different species and have typical antigenic capsid and haemagglutination (HA) properties [28, 30]. The mutations in different amino acid positions have led to the effective adaptation in the new hosts [30]. There are over five to six mutations in the VP2 residue of the CPV-2 and FPV and also 375 and 323 amino acid position regulates the pH functionality of HA [31, 32]. CPV-2a (Asn CPV-2a) replaced CPV 2 in 1980s in the USA and various European countries. CPV 2a can infect the cats which was not a feature of CPV 2. CPV 2a has been displaced by the CPV-2b (426Asp) which was first reported in USA in 1982 and CPV-2c (426Glu) variant in Italy [31, 33]. Although two variants, CPV-2a and 2b had been identified much earlier, however, the third variant CPV-2c had been recognized in early 2000 [33]. Thereafter it has been reported frequently from many different countries. In addition, new CPV-2a and new CPV-2b have also been documented due to non-synonymous substitution at 297 residues (Ser to Ala) of VP2 protein [34]. In India, CPV-2a has recently become the most prevailing antigenic type among all variants. Recent emergence of new antigenic variants that differ significantly from the current vaccine strains is a matter of concern for efficacy of vaccine [35].

3. Canine parvovirus enteritis (CPVE)

The CPV is of two types: CPV-1, commonly known as minute virus of canine and accountable for gastrointestinal and respiratory infection of dogs whereas, CPV-2,

most pathogenic type and is responsible for severe gastroenteritis/hemorrhagic gastroenteritis, in young puppies as well as adult dogs.

It is quite difficult to distinguish the clinical diseases caused by CPV-2 variants owing to its overlapping nature of signs and symptoms. These variants are believed to produce similar pathogenicity however; some studies showed that severity of clinical manifestations is influenced by variants of CPV-2 based on clinical, hematological, serological and histopathological examinations [36, 37].

Although puppies under 6 months of age are highly susceptible, adult dogs with insufficient immunity are also considered as high risk to the CPVE. CPV-2 can persist in the environment for more than a year, enabling susceptible dogs to pick up infection from CVP-2 contaminated feces, vomitus, or fomites. Although the feco-oral route is considered as primary path of disease transmission, infection through the oro-nasal route is also common in naive or under-immunized dogs due to ingestion of viruses shed in the vomitus or feces of CPV-2-infected animals [38]. However, direct contact or environmental contamination may also play a role [39]. Breed predisposition and seasonal prevalence of the disease are subject to considerable variations in wide geographical areas [40, 41].

Doberman, Rottweiler, and German shepherd (GS) dogs have been reported to be more susceptible to CPVE than other breeds [42]. Due to inherited immunodeficiency, the exotic breeds, German Sphered and Doberman, are more susceptible than the other breeds [43]. German shepherd has the highest CPV infection rate (70%) followed by the Doberman (55%) [44]. A cytokine bioassay revealed that the magnitude of TNF-α production by peripheral blood monocytes was greatest in dogs with a breed-related risk for CPVE. When compared to mixed breeds, highly susceptible breeds such as Rottweiler and Doberman Pinscher produce more TNF-α in response to LPS stimulation [45]. Increased TNF activity is predictive of mortality in naturally occurring CPVE infection in veterinary medicine [46]. Therefore, it has been hypothesized that dogs with a breed-related risk of developing CPVE, a disease associated with sepsis, would have a greater pro-inflammatory cytokine response to endotoxin [45].

The incubation period of CPV-2 infection ranges from 4 to 14 days. The infected dogs start to shed virus few days prior to the visible clinical signs and shedding of virus gradually declines 3–4 weeks postexposure [47]. Following entry into the body, the CPV-2 rapidly multiply in oropharyngeal lymph node, thymus and mesenteric lymph node, resulting in viremia within one week of exposure. After that, the virus attacks rapidly multiplying cells of crypts of intestine, epithelium of the tongue, oral cavity, bone marrow, and cardiac myocytes, besides lung, spleen, liver, and kidneys [48]. The key pathogenic event in CPV-2 infection is the virus-induced destruction of enterocyte, leading to mucosal barrier disruption, and villous atrophy. This causes profuse vomiting and hemorrhagic diarrhea, nutrient malabsorption, dehydration/hypovolemia, metabolic acidosis and/or alkalosis. The disruption of mucosal barrier allows bacterial translocation from intestinal compartment to systemic circulation, resulting in septicemia, endotoxemia, systemic inflammatory response syndrome as well as hypercoagulability [49]. The CPV-2 infection in the thymus and bone marrow precursor cells results in loss of thymic cortex and profound leucopenia, respectively [48]. Death may occur due to multi-organ failure when the affected dogs remain unattended [40, 49]. Previously, myocarditis was thought to be the acute cause of death in young puppies however, this form nowadays occurs rarely because of widespread CPV vaccination of dogs. The concurrent infections with parasitic, virus, or bacterial intestinal pathogens or stressors may aggravate the disease [50–52].

The degree of clinical manifestations may vary with age, breed, and immune status, duration of illness and virulence of virus. The clinical signs of dogs with

CPV infection are nonspecific in nature and resembles to gastritis and enteritis. The most notable clinical signs of CPVE are lethargy, depression, weakness, lack of appetence, bouts of vomiting, and diarrhea. The diarrhea is characterized by foul-smell and mucoid to purely hemorrhagic because slugging of intestinal mucosa and bleeding. The excessive loss of fluid during vomiting and diarrhea causes marked dehydration that results in development of hypovolemic shock. Occasionally, intussusception occurs due to intestinal dysmotility. Neurologic signs in puppies with CPVE may result from hypoxia secondary to myocarditis, hypoglycemia, or intracranial thrombosis or hemorrhages [52]. The bacterial translocation from intestine to systemic circulation can cause fever, systemic inflammatory response syndrome and septic shock with hypotension and organ failure [40, 48]. Apart from diarrhea, respiratory distress, pulmonary congestion and edema, alveolar and bronchiolar hemorrhage and convulsions are also occasionally manifested due to hypovolemia, endotoxic and septicemic shock [8, 53]. The malabsorbtion of nutrients and inadequate storage of glycogen in muscle and liver result in hypoglycemic encephalopathy which leads to seizures. On hospital admission, the prognosis is poor in CPVE dogs with intussusception, systemic inflammatory response syndrome and severe leucopenia.

4. Diagnostic approaches

4.1 Virus isolation

Virus isolation is considered as a gold standard for any viral disease diagnosis. In case of CPV-2 different cell lines like CRFK (Crandell Rees feline kidney), MDCK (Madin-Darby canine kidney) and A-72 are used for the isolation and propagation of the virus. The adapted virus causes distinct cytopathic effect in infected cell lines as cell rounding, aggregation, and necrosis of the affected cells. This requires the presence of special laboratory and is laborious [54].

4.2 Electron microscopy

It is an expensive technique for the detection of the virions by negative staining in the stool samples or culture isolated virus. Immunoelectron microscopy can also be done by using CPV-specific antibodies. The need of expensive electron microscope makes it out of reach for regular usage [55].

4.3 Haemagglutination test (HA)

The property of the CPV to cause agglutination of the pig, cat or rhesus monkey red blood cells at 4°C is used for detection of the CPV. The reciprocal of the maximum dilution of virus exhibiting ample agglutination of erythrocytes (mat formation) is designated as HA titer. The HA titer of more than 1:32 is usually considered as specific for CPV-2 [56].

4.4 Counter-immunoelectrophoresis (CIEP)

The use of electric current allows the rapid movement of antigen and antibody towards each other resulting into the formation of precipitation line quicker than

simple diffusion reaction. This technique is not commonly used but have been utilized for the prevalence of CPV infection in clinically suspected dogs [57].

4.5 Fluorescent antibody test (FAT)

In this test, an antibody tagged with fluorescent dye is employed for detection of specific CPV antigen. Mostly it is used as direct FAT for the diagnosis of CPVE but is not used routinely for diagnostic purpose [58].

4.6 Latex agglutination test (LAT)

This is a commonly used test utilizing antigen–antibody interactions employing specific antigen or antibody and is mostly useful under the field conditions. Here, the property of agglutination of polystyrene beads coated with either specific antigen or antibody on their surface is used with anti-CPV monoclonal and polyclonal antibody to detect CPV-2 in the stool samples. Earlier it has been used for both qualitative and quantitative evaluation of CPV in suspected dog feces. Also, a recombinant VP2 protein-based LAT for determination of immune status in dogs against CPV-2. Besides LAT, a slide agglutination inhibition test has been used to detect the presence of CPV-specific antibodies by utilizing the agglutination property of CPV-2 [59].

4.7 Slide inhibition test-slide agglutination test (SIT-SAT)

This method is developed for the detection of CPV-2. SIT is an antibody typing system based on the ability of viral antibodies to bind with the virus and prevents the virus from binding to RBC. SAT is used for antigen detection by serially diluting the clinical sample and then incubating it with a fixed amount of RBC containing virus surface receptors. The virus particles in the sample bind to the RBC and form a lattice that can be seen visually [60].

4.8 Enzyme-linked immunosorbent assay (ELISA)

It is an enzyme-based immunoassay involving antigen–antibody interactions to screen a large number of samples at a time. Recombinant VP2 protein-based indirect ELISAs has been developed to detect and quantify antibodies against CPV-2. Novel polyclonal antibody-based antigen capture ELISA using rabbit anti-CPV hyperimmune sera as capture antibody and guinea pig anti-CPV hyperimmune sera as detector antibody has been also developed. IgY-based ELISA comprising of the chicken egg yolk-derived has been developed for the detection of both antigen and antibodies. Different commercial ELISA kits are currently available for CPV-2 antigen and antibody detection [61].

4.9 Immunochromatographic (IC) assays

IC assays or Lateral flow assays are strip-based devices utilized for the detection of a target analyte in test samples. Colloidal gold nanoparticles are commonly used in synthesis of the probe (conjugate) in majority of these strip-based points of care assays. Different components used are the sample pad, conjugate pad, nitrocellulose membrane, absorbent pad and a plastic cassette. These tests are now used routinely for the parvovirus diagnosis in affected dogs. A number of lateral flow assay-based commercial kits are available for rapid detection of both CPV-2 antigen in feces and antibodies in

serum, which are also available in the market. These are helpful in the field and gives rapid results within 10–15 mins. Recombinant VP2 protein based immunochromatography tests has also been developed based on the rapid detection of CPV-2 [62].

4.10 Dot blot/dot-ELISA

It is an immunological test which uses charging of test antigen on to a nitrocellulose or PVDF membrane followed by detection using specific antibody against the antigen and an enzyme labeled secondary antibody which forms a color on addition of an insoluble substrate. It is helpful as on the spot assay for CPV diagnoses. It has been developed for detection of CPV-2 using hyperimmune sera raised against the whole virus/recombinant VP2 protein. Commercial dot ELISA kits are also available for evaluating IgM response against CPV-2 after vaccination or infection [63].

4.11 Polymerase chain reaction (PCR)

PCR is a molecular diagnostic assay which is used for the detection of viral nucleic acid and is relatively more sensitive than other conventional tests. Diverse antigenic types of the CPV can be distinguished by employing strain-specific primer or nested PCR or restriction enzyme analysis of the PCR. Also strain differentiation may be carried out with the help of oligonucleotide sequencing of the amplified gene [64].

4.12 Nucleic acid hybridization/dot blot

This has also been reported for the detection of CPV nucleic acid. Here hybridization with CPV-specific biotin or radiolabelled probe is carried out onto the CPV nucleic acid charged nitrocellulose paper or nylon membrane from suspected samples and then formation of color and band in the radiograph indicates the presence of the virus [65].

4.13 In situ hybridization assay

It uses an isotopic-labeled probe for both the detection and tracking of CPV nucleic acid in affected morbid tissue specimens thus,using more incubation time for development of the positive reaction [66].

4.14 Real-time polymerase chain reaction (qPCR)

This technique can be employed to quantitate CPV-2 in samples using either TaqMan probe technology or SYBR Green method. It is used for strain differentiation of concurrent infection using Multiplex Real-time PCR; and also, to differentiate vaccine strain from wild CPV strains. Different multiplex assays real-time PCR has been validated for the presence of CPV, FPV and PPV [67].

4.15 Amplification refractory mutation system PCR (ARMS-PCR)

It is used for the detection and typing of the known point mutations/single nucleotide polymorphism based on variable size of PCR-amplified products specific to a particular allele. In this PCR basically 2 pairs of primers are used (2 inner and 2 outer specific primers matching to individual allele type) in a single PCR tube and there are

no post-PCR protocols used as restriction enzyme digestion (PCR-RFLP) and sequencing therefore they provide an economical confirmation. ARMS-PCR is a well-known technique frequently employed for phenotypic association and single nucleotide polymorphism (SNP) studies. This has been used for CPV detection and its antigenic typing [54].

4.16 Peptide nucleic acid-based (PNA) Array

It contains a stable electrically neutral peptide backbone and the PNA-DNA hybridization assay are relatively more sensitive and specific than TaqMan-based real-time PCR for CPV differentiation [68].

4.17 Loop-mediated isothermal amplification assay (LAMP assay)

The assay is a sensitive and rapid technique used for amplification of DNA and thereby pathogen detection in an hour by using the DNA polymerase by autocycling strand displacement action by boiling at persistent temperature (60–65°C) in water bath. Usually, 2 sets of primers bind to 4 to 6 different regions of target viral DNA. LAMP has field application as there is no need for any thermocycler to carry out the target gene amplification. The amplification of VP2 gene of CPV-2 by LAMP assay has been developed. LAMP assay along with lateral flow dipstick (LFD) and LAMP-ELISA are also used for CPV DNA detection [69].

4.18 Insulated isothermal PCR method

It is a convection-based method using a hydrolysis probe for detection of CPV-2 and its antigenic variants. The reaction mixture is sequentially allowed to pass in an automatic manner through variable temperature zones in a capillary tube which undergoes thermocyclic phase to amplify the DNA and the probe hydrolysis produces optical output providing the result within an hour [70].

4.19 Polymerase spiral reaction (PSR)

This technique makes use of both conventional PCR and isothermal amplification as in LAMP and is completed within one and a half hour. Here mostly an exogenous sequence from an unrelated species or of botanical origin is incorporated at the 5′ end into the primer sequences used in PSR if a human or veterinary pathogen is targeted. PSR has been successfully used to detect all CPV antigenic variants with ten-fold higher sensitivity than traditional PCR [71].

4.20 Fluorescence melting curve analysis (FMCA)

It is a probe-based assay that uses melting curve analysis to detect and differentiate between CPV-2 variants. This assay consists of 2 TaqMan probes namely FAM labeled and HEX labeled. The FAM-labeled probe sequence is perfectly complementary to CPV-2a, with a 1 bp mismatch to CPV-2b and a 2 bp mismatch to CPV-2c. The HEX-labeled probe has complete complementarity with the original CPV-2 and a 1-bp mismatch with the other variants. This method is also capable of detecting samples containing more than one variant without sequencing [72].

Diagnosis	Specimen	Diagnostic assay used	Feature	Remarks
CPV antigen	Feces or rectal swab	ELISA	High specificity Low sensitivity	Feces or rectal swab
		Haemagglutination assay	Low-cost and rapid.	Sensitivity and specificity vary
Tissues or morbid samples	Necropsy specimens	Histopathology	Different histopathological techniques and IHC may be used.	Differential diagnosis with other enteric infections
Viral DNA	Feces or rectal swab or any tissue	Polymerase chain reaction (PCR);qPCR	Efficient in diagnosing even minute amount of viral genome, can be quantified; Antigenic typing	Sensitivity and specificity vary. Vaccine virus shedding occurs upto weeks after immunization leading to false positives results. Inhibitory components may lead to false negative results.
Virus	Feces or rectal swab or any tissue	Virus isolation	Confirmatory diagnosis	Requires special facility
Virus particles	Feces or rectal swab or any tissue	Electron microscopy	Confirmatory diagnosis	Requires special facility, expensive

Table 1.
Summary of the different types of diagnostic assays for CPVE diagnosis.

Sl. No.	Test	Company	Principle	Reference
1	SNAP parvo antigen test	IDEXX, United States	ELISA	[76]
2.	Rapid Immunochromatographic (IC) strip test	ADDBIO, Korea	Immunochromatography test	[43, 77, 78]
3.	Witness Parvo Test Kit	Zoetis, United states	Rapid Immuno Migration (RIM™) technology.	[79]
4.	Fassisi® Parvo	Fassisi, Gottingen, Germany	Lateral flow immunoassays	[80]
5.	FASTest parvo card	Vet lab, UK	Lateral flow immunoassays	[55]
6.	4 CPV Antigen Rapid Test Kit	Ubio Biotechnology systems Pvt. Ltd., India	Lateral flow immunoassays	[79]
7.	Anigen Rapid CPV Ag Test Kit®	Bionote, Dongtan, South Korea	Lateral flow immunoassays	[80]
8.	ImmunoRun CPV antigen detection kit	Biogal- Galed labs, Israel	Immunochromatographic assay	[79]
9.	Primagnost® Parvo H + K	Dechra, Aulendorf, Germany	Lateral flow immunoassays	[80]

Sl. No.	Test	Company	Principle	Reference
10.	Canine Parvovirus & Distemper IgMAntibody Test Kit	Biogal Galed Laboratories Acs Ltd., Israel	Immunocomb	[79]
11.	Vetexpert Rapid Test CPV Ag®	Vetexpert, Vienna, Austria	Lateral flow immunoassays	[80]

Table 2.
List of commericially available kits for CPV-2 detection.

4.21 DNA aptamers

Aptamers emerged as a good alternative to antibodies as affinity reagents. Recently, ssDNA aptamers that specifically bind with the recombinant VP2 (rVP2) protein of CPV-2 with affinity in the nanomolar range have been reported. The ssDNA aptamers specific to CPV-2 (rVP-2) were selected by the Systematic evolution of ligands through exponential enrichment (SELEX) method and their target binding was assessed by dot blot and enzyme-linked oligonucleotide assay (ELONA). Aptamers with high binding affinity and specificity against rVP-2 could be employed in diagnostics for rapid detection of CPV-2 [73].

4.22 Nucleotide sequencing

It is primarily used for most viral genome identification and confirmation. Thus, considered as a gold standard for the antigenic typing of CPV variants. The amplified PCR product is either directly sequenced or cloned which is sequenced in a sequencer utilizing apt primers. The sequence data is analyzed using the appropriate bioinformatics database. Either nucleotide or amino acid sequence data or even both could be employed to recognize the evolutionary analysis of CPV-2 isolates from different geographical sites [74].

4.23 Biosensor

It is an analytical device which detects the DNA/RNA/protein/enzymes and alters it to the detectable electrical signals. A biosensor for CPV detection has been established by means of quartz crystal microbalance biosensor and ProLinker B [75]. Summary of different types of diagnostic assays are listed in the **Table 1**.

4.24 Commercially available kits

Commercially available kits are mostly based on antigen–antibody reactions, such as ELISA, dot ELISA, and immunochromatographic strip-based assays (**Table 2**).

5. Treatment of CPV-2 infection

5.1 Recent advances in therapeutic management of CPVE

In absence of effective and appropriate antiviral drugs, the most universal therapeutic regimen for CPVE is supportive and symptomatic care until vomiting

and diarrhea have resolved. Because of long-term illness of CPVE infected dogs, the challenges faced by the pet owners are cost of treatment and hospitalization. In private practice settings, the treatment cost may be huge, indicating that financial constraints may be a factor in disease-related euthanasia [81]. Therefore, fatality of CPVE is documented more in socioeconomically underprivileged areas, where level of education and financial opportunity for care and vaccination are not adequate [82]. Although the survival rate of CPVE in hospitalized and outpatient dogs is debatable, a recent prospective, randomized trial found no significant differences in survival (90% vs. 80%, P = 0.66) or duration of hospitalization (4.6d vs. 3.8d, P = 0.20) between inpatient and outpatient dogs [83]. However, given the possible risks of long-term hypoglycemia and leukopenia, aspiration pneumonia, edema, and intussusception in CPVE dogs, hospitalization appears to be the better option over outpatient treatment [84].

The principal components of supportive and symptomatic therapy include 1) fluid therapy and oncotic support, 2) antibiotics, 3) antiemetics, and 4) nutritional support. A wide range of other treatment measures including, though not limited to, antiviral treatments and pain management have been assessed in the past or are currently under investigation regarding their potential utility in CPVE.

5.2 Management of fluid and electrolyte imbalance and oncotic support

The development of severe hypovolemia is the first impact of pathophysiology in dogs with CPVE, hence re-establishment of the circulating volume is the utmost need [85]. The hypokalemia, hypochloremic metabolic alkalosis, hypoglycemia, hypoproteinemia and loss of oncotic pressure in circulation are the major fluid and electrolyte abnormalities during episode of diarrhea and vomition in acute CPVE [86]. The most aggressive therapies consisting of administration of intravenous (IV) fluids to restore intravascular fluid volume status, replenish interstitial fluid losses, maintenance of hydration and oncotic support. A balanced isotonic crystalloid solution (eg, Lactated Ringers) should be used for initial restoration of intravascular volume and rehydration, with a rate titrated to improve perfusion parameters such as capillary refill time, mucosal color, pulse character, and mean arterial pressure or lactate concentrations. Apart from fluid administration, potassium need to be supplemented in hypokalemic patients whereas, 25% dextrose at the dose rate of 1-2 mL/Kg body weight followed by addition of 2.5–5% dextrose in the crystalloid fluids will be required for hypoglycemic patients with blood glucose level < 60 mg/dL. Initially, the fluid is administered at the dose rate of 80–90 mL/kg with a boluses of 15–20 mL/kg over 15–20 minutes to counter the hypovolemic shock and, to improve the fluid perfusion. After that, the maintenance dose for daily fluid depends on the body weight (kg) and percent of dehydration. The volume (L) required to correct the daily fluid loss is calculated as body weight (Kg) × % dehydration. Generally, 40–60 mL fluid for each kg body weight is considered as ideal maintenance dose. Since fluid absorption through subcutaneous route is impaired in hypovolemic patients, intravenous access is considered as choice of fluid treatment. However, intraosseous or jugular catheter are considered as appropriate option in severe hypovolemic or interstitially dehydrated patients [87].

In CPVE, protein loosing enteropathy attributes to pronounced hypoalbuminemia (<2 g/dL) and/or hypoproteinemia (<4 g/dL) resulting in peripheral edema, pleural or abdominal effusions [88]. In that case, provision of oncotic support in the form of either natural or synthetic colloids are very important to minimize the morbidity

and mortality of patients [89]. For correction of hypoalbuminemia, fresh plasma (20 mL/kg) or fresh-frozen plasma (6.6–11 mL/kg IV or 3 doses administered intraperitoneally 12 hours apart) and canine-specific albumin concentrate are used [90]. The concentrated human albumin products can also be used but the risk of immune reaction is the major limitation. If further oncotic support is required, hydroxyethyl starch (20–30 mL/kg/d) can be given, depending on clinician choice [6]. Sometimes, administrations of whole blood (20 mL/kg, within 4 hours) or packed RBCs are needed in severe anemic dogs with CPVE.

5.3 Antiemetic treatment

Apart from fluid and electrolyte imbalance, emesis is another clinical manifestation in CPVE. So, antiemetic treatment is warranted in CPVE otherwise persistent vomition may enhance the duration of hospital stay and further aggravates the condition of patient. The clinical efficacy of number of antiemetics in CPVE had been investigated with varying degree of results. The earlier studies showed that metoclopramide, a dopaminergic antagonist, was found to be effective in reducing episode of vomition by exerting a prokinetic effect in the upper intestinal tract and blocking the chemoreceptor trigger zone when administered as a bolus or as a constant-rate infusion in dogs. The ondasetron or dolasetron, the serotonin receptor antagonists, are also found effective in reducing the number of vomiting events [85]. Recently, a substantial antiemetic effect of maropitant, an antagonist of neurokinin1 receptors, by stimulation of either central or peripheral emetic pathways has been reported in dogs however, the efficacy of maropitant in CPVE has yet to be thoroughly investigated [91]. The administration of maropitant once daily, singly or in combination with metoclopramide, is very effective in reducing vomition in CPVE [5].

5.4 Antimicrobial treatment

Translocation of bacteria from intestinal compartment to systemic circulation is very common in CPVE because of villous collapse and disruption of the mucosal barrier. The translocation with concurrent marked neutropenia leads to a high risk of septicemia and endotoxemia. Additionally, hypotension from fluid loss and sepsis make dogs with CPVE at high risk of developing acute kidney injury. Therefore, parenteral administration of broad-spectrum bactericidal antibiotics is necessary in dogs with CPVE. Ampicillin and cefoxitin as single-agent treatments or in combination with enrofloxacin are the choice antimicrobials against Gram-positive and negative bacteria [85]. Aminoglycosides may also be considered in well-hydrated animals otherwise it may be avoided due to its inherent risk of nephrotoxicity. Puppies with CPVE often have comorbidities, including gastrointestinal parasitism. Hence, antiparasite therapy should be initiated once the puppy can tolerate oral therapies [6].

5.5 Nutritional support and pain management

Restoration of early mucosal integrity and prevention of bacterial translocation from gut compartment to systemic circulation are very important for faster recovery of dogs with CPVE. Enteral feeding is reported to improve the mucosal integrity and faster repair, resulting in lower possibilities for bacterial translocation [8]. In earlier study, it was demonstrated that early enteral nutrition via

nasoesophageal catheter starting 12 hours post-admission led to clinical improvement, significant weight gain, and improved gut barrier function was more early as compared to withholding of the traditional food until cessation of vomiting for 12 hours [92].

Severe vomition, enteritis, and or concurrent intussusception in CPVE are the possible reasons for abdominal pain. Hence, analgesic treatment to reduce visceral pain is one the important aspect in therapeutic management in CPVE. Partial mu-agonists such as buprenorphine (0.01–0.02 mg/kg IV every 8 hours) or an agonist–antagonist such as butorphanol (0.1–0.2 mg/kg/h) are the preferred analgesics over the pure mu agonists as opioid analgesics can promote ileus and vomiting. The α-2 agonists that promote extreme vasoconstriction and limit gastrointestinal perfusion, and non-steroidal anti-inflammatory drugs that impair gastrointestinal and renal perfusion, both are not indicated [93].

5.6 Antiviral drugs

Like other viral infections, prophylaxis is the cornerstone for prevention of CPV in dogs. Although, an adequate number of killed and live CPV vaccines are marketed by pharmaceuticals but vaccines sometimes fail to protect completely due to poorly responding breeds (Rottweilers and Doberman pinschers), variation in genetic makeup of field and vaccine viruses, interference by presence of maternal antibodies and adjunct factors [94]. Therefore, development of some suitable antiviral drugs is utmost important for effective management of the CPVE in its acute illness stage. Till now, only few antiviral drugs have been evaluated for its clinical efficacy against CPVE. In an earlier placebo-control study, the therapeutic efficacy of Oseltamivir, a neuraminidase inhibitor, in CPVE had been evaluated and noted that Oseltamivir did not produce any additional benefit in terms of reduction of mortality or duration of hospitalization except some improvements in body weight and hemogram in dogs with CPV-illness [95]. In another study on naturally infected dogs, a promising anti-CPV activity of recombinant feline interferon-ω (rFeIFN-ω) has been recorded as compared to placebo-group. The intravenous administration of rFeIFN-ω at the dose rate of 2.5 mU/kg daily for consecutive three days remarkably reduced the clinical symptoms and mortality [96, 97]. Although the drug is currently available for use in Europe and Australia, the high price and frequent non-availability are major limitations. Recently, another antivital drug, Acyclovir, guanine analogue commonly used to treat herpes simplex virus infection, have been shown to improve the disease conditions [98]. Further, an *in-vitro* study on A72 cell line showed that 9-(2- hydroxyethomethyl) guanine phosphoromonomorpholidate (ACV PMMPD), a phosphorimidate analogue of acyclovir inhibits CPV-2 replication with exhibiting 50% inhibitory concentrations (IC50s) in the low-micromolar range (50 μM) [99]. Recently, broad-spectrum anti-CPV activity of some anti-parasitic drugs such as Nitazoxanide, Closantel Sodium, and Closantel have also been shown using F81 cells [100].

5.7 Passive immunotherapy

Passive immunization with specific antibodies against enteric viral infections in animals confers significant protection, reduces diarrhea and virus shedding and increase survival rates [101]. Thus, the of immunotherapeutics in viral infections is promising treatment approach because of lower adverse effects as well as no chance of any resistance as in antiviral drugs. The passive immunization by means of oral

or intravenous administration of IgY specific for CPV-2 shows the protective effect in dogs challenged with the virus [102]. The reduction of clinical scores, duration of symptoms and mortality and improvement of body weight gain has been reported by anti-CPV-2 IgY therapy in experimentally produced CPVE [103]. Recent study reported that chicken IgY- single chain fragment variables (scFv) generated against the virus capsid protein could be a promising therapeutic target against CPV [104, 105]. Aside from IgY, the neutralization of CPV by anti-feline panleukopenia virus antibodies is also reported from an *in-vitro* study [106]. However, the prospective, randomized, placebo-controlled, double-blinded study on CPVE did not produce substantial benefits when compared with placebo group [86]. Apart from above therapeutics, plasma therapy is also another option. Although the administration of CPV-hyperimmune plasma is reported to decrease the clinical signs and improve the survival rate in dogs under experimental conditions, however the findings remain inconclusive in natural cases [107]. Another study showed that lyophilized IgG treatment reduced clinical signs and duration of hospitalization of dogs naturally infected with CPV [108].

5.8 Immunomodulators

The key physiopathological alterations of CPVE are destruction of intestinal crypts, neutropenia, secondary bacterial translocation, immunosuppression due to thymus atrophy, sepsis and systemic inflammatory response syndrome in puppies [6, 109]. Therefore, immunomodulators could be an option to enhance therapeutic efficacy of supportive treatment. A recent study demonstrated that subcutaneous administration of human dialyzable leukocyte extract-h (hDLE) along with supportive therapy in puppies with CPVE significantly increased the leukogram and reduced the clinical score, duration of hospitalization, mortality as compared to supportive therapy alone [110].

5.9 Cytokines based therapeutics

5.9.1 Granulocyte colony-stimulating factor

Leukopenia is one of the most important prognostic indicators of mortality in dogs with CPVE. Hence, stimulation of bone marrow and improvement of leukogram in peripheral circulation are considered as strategic approaches to reduce the CPVE associated mortality. Enhancement of endogenous canine G-CSF (cG-CSF) concentrations by exogenous administration of human G-CSF (hGCSF) and cG-CSF is reported to stimulate bone marrow, resulting in improvement of neutrophil counts in puppies with CPV infection [111]. However, the use of hG-CSF and cG-CSF may not necessarily improve survival [112, 113].

5.9.2 Interferons and biological response modifiers

The interferon (IFN)-ω, a type I IFN (similar to IFN-α), is known for its antiviral, anti-proliferation, and antitumor activities. A notable therapeutic effect of rIFN-ω on CPV-infected dogs is reported [114]. Additionally, the promising therapeutic potential of other type I (IFN-α, IFN-β, IFN-ε, and IFN-κ) and III (IFN-λ) IFNs in CPVE has also been reported [115].

Recently, anti-CPV activity of the serum derived transfer factors (TFs), low molecular weight (<5000 daltons) biological response modifiers has been

documented. It imparts therapeutic benefit in CPVE by altering the cytokine response of the host [116].

5.10 Probiotics

Probiotics, primarily comprised of live microorganisms in fermented foods, protect gut from acute diarrhea through adherence and colonization on gut mucosa [117]. Therapeutic efficacy of probiotics has been verified in dogs with CPV associated illnesses [118]. In an earlier study, oral administration of probiotic preparations as an adjunct therapy to young dogs with CPVE has shown faster resolution of clinical signs, improved leukogram and decreased mortality as compared to supportive treatment alone [119]; whereas, no benefit with respect to length of hospital stay or case fatality was recorded in other study [120].

5.11 Antioxidants

The disturbance in oxidant/antioxidant equilibrium is evident in CPV-gastroenteritis and oxidative stress is believed to link with pathogenesis of CPVE [121]. Hence, addition of antioxidants in supportive therapy has emerged as a promising therapeutic option to improve the response of treatment in viral diseases. Treatment with *N*-acetylcysteine (NAC), a precursor to glutathione and the body's primary cellular antioxidant, along with supportive therapy markedly improved the leukogram in dogs with CPVE when compared with supportive therapy alone [122].

5.12 Herbals

An interest in natural products including herbs, plants and their extracts/metabolites as antiviral drug candidates has increased in the last few decades especially due to rising emergence of antimicrobial resistance globally and potential side-effects of many antimicrobials [123]. Very recently, anti-parvoviral activity of propolis, a traditional Chinese medicine, prepared from honeybee hives has been documented [124]. The *in vitro* study on PK-15 cells showed that ferulic acid (FA), an important component of propolis attenuates the replication of porcine parvovirus by blocking proapoptotic factors (Bid, Bcl-2 and Mcl-1), and inhibiting the mitochondria-mediated response by hindering the activation of the Bid-related signaling pathway. The FA may serve as potential antiviral against CPV [124].

5.13 Fecal microbiota transplantation

Alteration in the gut microbiome is reported in enteric viral diseases including CPVE and other gastrointestinal diseases in dogs [125]. The disruption of gut microbiota leads to impediment in the enterocyte nutrition, immune regulation, protective barrier function, and gastrointestinal motility [126]. Therefore, restoration or re-establishment of the microbiota could have a good interest therapeutically. Recently, a randomized clinical trial showed that administration of fecal microbiota (10 g feces diluted in 10 mL of sterile 0.9% saline) obtained from healthy donor rectally at 6–12 hours post-admission caused faster resolution of diarrhea, shortened the duration of hospitalization and reduced the mortality in young dogs with CPVE when compared with standard therapy alone [126].

6. Prevention

A modified live virus (MLV) and an inactivated vaccine are the two types of CPV-2 vaccines currently available [94]. Administration of the vaccine should start at 6 to 8 weeks of age and then every 2–4 weeks until 16 weeks of age or older. For dogs that are 16 weeks or older, 2 doses of vaccination are recommended with an interval of 2–4 weeks [127]. A recombinant vaccine based on virus-like particles (VLPs) is being developed, which has the advantage of becoming highly immunogenic and safe [128]. Peptide vaccines containing major antigen neutralizing region N terminal of VP2 are also under developmental stage [129]. A single-dose vaccination of Vaccinia virus encoding CPV2-VP2 elicited substantial antibody responses and provided comparable protection for dogs with attenuated CPV2 vaccine. This vaccine could be used as a promising vaccine candidate to prevent CPV-2 infection in dogs [130].

7. Conclusion

CPV-2 is one of the most significant viral enteropathogens of canines causing high morbidity and mortality and manifested by vomition and severe acute haemorhagic gastroenteritis. Prompt symptomatic therapy will increase survivability of infected puppies but vaccination is best way to prevent the disease in dogs. Despite the pups are protected through vaccination from the pregnant bitch, it is more vulunerable to CPV-2 infection as maternal antibody titers started declining. Despite the availability of high sensitive and specific diagnostic approaches and the effective prophylactics such as modified live virus and inactivated vaccines, a large number of outbreaks are still reported in wide geographical areas across the globe in both vaccinated and unvaccinated dogs. The future studies should be taken up towards vaccination failures, occurrence of CPV-2 in different canine species and the emergence of antigenic variants of the CPV-2 involved in the outbreaks.

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Section 2

New Advances in Canine Cancer

Chapter 4

Immunology of Canine Melanoma

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Abstract

Malignant melanoma is one of the most important tumors in dogs and is highly metastatic and aggressive disease. In recent years, molecular knowledge regarding canine melanoma has increased, and some chromosomal imbalances and tyrosine kinase pathways have been identified to be dysregulated. Mxoreover, canine melanoma is an immunogenic tumor that provides opportunities to administer immunotherapy to the patient. Podoplanin and chondroitin sulfate proteoglycan-4 (CSPG4) are markers against which monoclonal antibodies have been developed and tested in dogs *in vivo* with promising results. Owing to the importance of canine melanoma in the veterinary oncology field, this chapter reviews the most important aspects related to immunological involvement in the prognosis and treatment of canine melanoma.

Keywords: dogs, immunotherapy, immune system, melanocytic tumors, T-cells

1. Introduction

Canine melanoma is an aggressive tumor that originates from melanocytes in different sites in the animal body, including the oral cavity. Melanoma of the oral cavity is a highly aggressive disease and is the most prevalent cancer in the oral cavity [1–9]. According to the Oncology Pathology Working Group (OPWG) consensus, some of the most common histologic features of melanoma include intracytoplasmic melanin, variable cell morphology, junctional activity, pagetoid growth, presence of neoplastic cells at the mucosal–submucosal junction, and finely stippled to vesiculated nucleus with a prominent central nucleolus ("owl's eye") [9]. Distant metastasis, lymphatic invasion, nuclear atypia, mitotic index, tumor size/volume, and tumor score are also related to poor prognosis [6, 9, 10].

Canine melanoma is an immunogenic tumor, and investigations of different aspects associated with immune cells in tumor development and progression have been reported in the literature [1–10]. Moreover, genomic and transcriptomic data from canine oral melanoma have revealed several pathways associated with inflammatory processes, including T-helper cell differentiation [1]. Thus, evaluation of the immune components of canine melanoma is pivotal for a better understanding of tumor biology and for developing new therapies. Therefore, this chapter reviews the association between the immune system and canine melanoma development, prognosis, and treatment.

2. Literature review

2.1 Tumor etiology

Canine oral melanoma is the most frequently diagnosed malignant tumor in dogs [2]. Oral melanoma is associated with a high local infiltration, metastatic rate, and poor prognosis. Biological behavior and presentation of melanoma vary remarkably and are influenced by anatomic site, stage, and histological features [1, 2]. Increasing age, with no relation to sex, is also a determinant for canine oral melanoma aggressiveness [2, 3, 6].

The etiology of canine oral melanoma is multifactorial, including environmental and genetic factors [6]. In cutaneous melanoma, hairy skin and sunlight exposure could be considered risk factors [2]. However, sunlight exposure cannot be considered a risk factor for oral melanoma. Chronic inflammation or trauma, deep bacterial infections, intralesional necrosis, chemical exposure, and burns are factors associated with canine melanoma development [6, 8].

Highly pigmented oral mucosa and purebred dogs, such as Airedale Terrier, Boston Terrier, Boxer, Chihuahua, Chow, Cocker Spaniel, Doberman Pinscher, English Springer Spaniel, Golden Retriever, Irish Setter, Miniature Schnauzer, Scottish Terrier, Poodles, Beauce Shepherds, Rottweilers, and Labrador Retrievers, are predisposed to melanocytic tumors, including oral melanoma [3–5]. This suggests that melanoma in dogs may have genetic factors [7, 8].

3. Melanoma clinical signs and diagnosis

Clinical signs of canine oral melanoma include halitosis, dysphagia, anorexia, weight loss, facial swelling or swelling of the lymph nodes, drooling, panting, loss of teeth, and oral and facial pain, which may induce the dog to avoid being touched. Dogs affected by oral melanoma can also be asymptomatic; in these cases, the owner or veterinarian may discover the oral mass only during routine examination. The owner may also notice blood in the water or food bowl or that oozing from the mouth [10–13].

The diagnostic methods include biopsy of the tumor, followed by histopathological examination. Immunohistochemical tests can provide a reliable and definitive diagnosis [7]. The diagnosis may be confirmed by cytology of a fine-needle aspirate [10]. Immunofluorescence can also be used to distinguish melanomas from melanocytomas [14]. However, other tests, such as skull radiographs, chest X-rays, mandibular lymph node samples, abdominal ultrasound, serum biochemistry, and complete blood count, are crucial for a better patient health overview, metastasis detection, and safer treatment choice [15].

4. Prognostic factors for canine melanomas

A histological and epidemiological study including 384 cases of melanocytic tumors comprised 19% oral tumors, of which 59% were malignant. In contrast, analyses of melanocytic tumors of the skin identified only 12% of patients with malignant tumor [6]. Regarding the prognostic criteria for canine melanoma, mitotic

index, nuclear atypia, tumor volume, the presence of metastasis, and the presence of deep inflammation or intralesional necrosis remain pivotal when determining patient outcome [6, 9].

A previous study of 67 oral melanoma samples suggested that free surgical margins and chemotherapy with carboplatin increased patient survival [16]. Another study indicated that melanocytic tumors were more common in middle-aged dogs with dark hair and undefined breeds. Histological analysis also revealed the prevalence of epithelioid cells [17]. Another study evaluated 338 canine oral melanoma cases, with an overrepresentation of breeds, such as Chow, Golden Retriever, and Pekingese/Poodle mix, but with no mention of hair color. Histological evaluation suggested the presence of polygonal and spindle cells [18]. However, the mismatched results suggested that epidemiological data for canine oral melanoma require detailed evaluation.

5. Immune system and cancer

The relationship between the immune system and the tumor's ability to evade it is a hallmark of cancer [19]. An increasing number of studies have investigated the role of the immune system in tumor progression and its therapeutic potential for various tumors. The immune system comprises several cell types, and recognizes and eliminates biological, chemical, and physical dangers from the body via a series of humoral and cellular pathways and interactions [20]. In contrast, a neoplastic cell is an autologous cell that harbors suppression and overexpression of certain genes, mainly tumor suppressor genes and oncogenes, respectively. Neoplastic cells express proteins that are not recognized by the immune system; thus, receptors cannot indicate to the immune system that the cell is abnormal to the organism. Therefore, the immune system is an important factor in the body to prevent tumor progression. Taken together, it is necessary for the tumor to employ mechanisms to evade the antitumor immune response.

A neoplastic cell proliferates in an uncontrolled manner and each daughter cell may be the same as its mother cell; however, many cells accumulate new genetic defects, generating intratumor heterogeneity. This allows the neoplasm to adapt to different adversities for its progression, such as the antitumor action of the immune system. This process of interaction between neoplastic cells, immune cells, and the entire tumor microenvironment presents a complex cell-cell relationship. The most accepted "tumor immunoediting theory" that explains the events of this interaction is divided into the following three phases [21]:

1. Elimination: Cells of the immune system recognize and eliminate pre-neoplastic and neoplastic cells. In this phase, complete elimination of cells may occur, ending tumor initiation; however, some cells may escape surveillance of the immune system and proceed with carcinogenesis.

2. Balance: Tumor growth is equivalent to elimination of neoplastic cells by the immune system. In this process, tumor cells with lower immunogenicity, that is, those capable of evading the immune system, are selected.

3. Escape: Tumor growth occurs due to the reduction in/inability of the immune system to eliminate neoplastic cells and the rapid growth of neoplastic cells.

6. Tumor immune evasion mechanisms

The immune system can react with agents that are harmful to the body in several ways. Innate immunity, responsible for this first interaction, can corroborate the antitumor activity with a nonspecific reaction to the tumor [22], such as treatment with Bacillus de Calmette and Guerin of human melanomas [23], that initially induces a nonspecific response to this agent, but later affects tumor progression. In contrast, innate immunity, which is primarily responsible for the antitumor activity of the immune system, is triggered by recognition of tumor cells by CD8+ T cells. They eliminate tumor cells by various pathways, such as perforin/granzyme or by induction of apoptotic pathways [22].

The interaction between the immune system, neoplastic cells, and tumor microenvironment is extremely complex and orchestrated by numerous regulatory factors, whether intrinsic or extrinsic to neoplastic cells. Neoplastic cells employ several tactics for evading the immune system, and consequently, the permanence of the escape phase in the immunoediting process. Cells use several mechanisms to succeed in this process, ranging from the non-recognition of cells as non-self to the production of inhibitory factors and exhaustion of immune system cells, including the activation of bone marrow-derived suppressor cells, activation of regulatory T cells (Tregs), alteration of dendritic cell functions, production of cytokines, and non-recognition of cells due to non-expression of histocompatibility molecules [24].

7. Myeloid-derived suppressor cells (MDSCs)

An immune cell population responsible for suppression of the immune system in tumors is myeloid-derived suppressor cells (MDSCs). These cells are formed by populations of monocytes and immature granulocytes arising from the bone marrow during pathological conditions [25]. Numerous studies have indicated an increase in the number of cells of this type in cancer in humans and mice [26]; but, its role in dogs is unclear [27].

In humans, MDSCs are responsible for immunosuppression of the tumor site, allowing a more invasive and metastatic characteristic through the production of metalloproteinases [28, 29]. A study [25] on the immunosuppressive action of MDSCs indicated that MDSCs can suppress natural killer cells, dendritic cells, and T lymphocytes, in addition to potentiating the effects of Tregs by producing reactive oxygen species and inducible nitric oxide synthase (iNOs). MDSCs also produce immunoregulatory cytokines, such as TGF-β and IL-10 and decrease the expression of IL-12, which is responsible for the activation of T cells [30].

8. Induction of regulatory T cells by tumors

Tregs, in addition to natural killer cells, are CD4 T cells capable of directly suppressing CD4+ and CD8+ T cells. Thus, the presence of Tregs in the tumor microenvironment may be involved in a decrease in the immune response, and many studies have demonstrated that an increase in these cells is correlated with worse prognosis. Muir et al. [31] demonstrated the prognostic role of Tregs in canine lymphomas, with dogs with higher levels of Tregs showing worse prognoses. A study by Curiel et al. [32] also demonstrated that an increase in the number of these cells in ovarian carcinoma predisposes dogs to a lower survival rate.

9. Impaired dendritic cell activation and function

Dendritic cells are the main antigen-presenting cells (APC) that play an important role in the activation of CD8+ T cells and NK cells and are considered the main tumor surveillance cells. Neoplastic cells employ mechanisms to escape the immune system. The activation and alteration of dendritic cells, such as an increase in the production of IL-10 that antagonizes their antigen-presenting action, inactivates dendritic cells and reduces their ability to stimulate other cells. They induce anergy to specific antigens. Another study suggested that the release of IL-6 in the tumor environment keeps the dendritic cells immature, worsening the prognosis of patients with several tumors, as in the case of melanoma [33].

The importance of dendritic cells has been further explored in antitumor immunotherapy, including the use of dendritic cells in the production of antitumor vaccines [34].

10. Failure of tumor cells to activate immune system

Tumors can also prevent the activation of the immune system by decreasing the expression of type I and II histocompatibility molecules [27]. The expression of co-inhibitory molecules, such as CD73 and programmed death ligand-1 (PD-L1) allows tumors to evade the immune system. The programmed cell death protein-1/programmed death ligand-1 (PD-1/PD-L1) pathway promotes apoptosis of CD8+ T lymphocytes [35]. Whereas cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) is a co-inhibitor expressed by T cells that negatively regulates the activation of T cells. Therefore, the tumor can avoid the immune system because the immune cells are unable to recognize neoplastic cells either by apoptosis or exhaustion mechanisms. Many studies have focused on these pathways to inhibit tumor cells, and thus, restore the recognition and activation of the immune system.

11. Cytokines

Neoplastic cells produce different cytokines that alter the tumor microenvironment. The tumor microenvironment has an extremely delicate balance between antitumor effects and the origin of tumors arising from pro-inflammatory activity that is dependent on the mediator cells [36]. Therefore, the cytokines may have antitumor and protumor effects, depending on the interrelationship between the

Main cytokines	Main activity
IL-2	Induces T-cell proliferation and differentiation into effector T cells Increases cytotoxicity of "Natural killer" cells Induces proliferation of B lymphocytes
IL-3	Promotes production/differentiation and proliferation of macrophages, monocytes, granulocytes, and dendritic cells
IL-4	Induces CD4+ T lymphocyte differentiation in cells with Th2 phenotype Increases production of MHC-II Induces growth and differentiation of B lymphocytes
IL-6	Pro-inflammatory and anti-apoptotic cytokine that may contribute to tumor development associated with chronic inflammation

Main cytokines	Main activity
IL-8	Chemotactic and activating factor of neutrophils and T lymphocytes
IL-10	Immunosuppressive cytokine produced by activated dendritic cells, macrophages, and T cells
IL-11	Stimulates proliferation of hematopoietic stem cells Induces megakaryocyte maturation
IL-12	Stimulates synthesis of IFN-γ and TNF-α by T cells and natural killer cells decreasing angiogenesis
IFN-α, IFN-β	Induces apoptosis of tumor cells Activation of natural killer cells Inhibits tumor angiogenesis
IFN-γ	Promotes CD4+ T cell differentiation to Th1 phenotype Activates macrophages Modulates MHC I/II expression
TNF-α	Stimulates angiogenesis and metastasis of some tumors Important pro-inflammatory cytokine
TGF-β	Immunosuppressive cytokine Inhibits macrophage activation and B lymphocyte growth High expression in various tumors

Adapted from the small animal clinical oncology [27].

Table 1.
Main cytokines relevant to tumor immunotherapy.

tumor and constituents of the tumor microenvironment [37]. Thus, the use of cytokines as therapeutic agents is quite complex, as their action is dependent on the status acquired in the tumor microenvironment. Lin and Karin [37] also showed that chronic inflammation leads to the production of several cytokines that allow tumor development, highlighting that the interaction between cells present in the tumor microenvironment determines the effects of the released cytokines. Catchpole et al. [38], for example, demonstrated an increase in the production of IL-10 and TGF-β and a decrease in that of IL-2, IL-4, and IFN-γ in the lymph nodes with melanoma metastasis. Each cytokine has different actions on different cells, and its effects can either help in carcinogenesis or confer antitumor activity (**Table 1**).

12. State-of-the-art of immunology in canine melanoma

Canine melanoma is an immunogenic tumor, and investigations of different aspects associated with immune cells in tumor development and progression have been reported in the literature [39–41]. Moreover, some studies have evaluated different cancer immunology aspects of canine melanoma, podoplanin [42], and chondroitin sulfate proteoglycan-4 (CSPG4) [40]. Podoplanin is a type I transmembrane protein that is expressed in different cells of the immune system, including lymphatic endothelial cells. Podoplanin overexpression has been investigated in several cancers, including canine melanomas. Since the development and application of a monoclonal antibody against podoplanin, these markers have been recognized as important for canine melanoma immunotherapy [43]. CSPG4 has become very important for canine melanoma owing to the number of vaccines produced against this protein [39, 44, 45].

Its expression has been reported in canine melanoma, and different clinical trials based on vaccines or electrogene therapy have been conducted [39, 44, 45]. A review of the PubMed database for the past ten years is provided in **Table 2** summarizing clinical trials involving dogs with melanoma treated with immunotherapies.

In addition to evaluating immunotargets for canine melanoma, several studies have investigated the association between immune markers and melanoma prognosis,

Reference	Number of subjects and cancer type	Manuscript goal	Manuscript summary
Riccardo et al. [39]	80 oral melanomas	Evaluate the clinical efficacy of a vaccine targeting tumor antigen chondroitin sulfate proteoglycan (CSPG)4.	Authors developed a hybrid DNA vaccine against human/dog CSPG4 chimera, with results indicating a safe and immunogenic vaccine, prolonging the survival of melanoma-affected patients, and promising in clinical routine.
Saellstrom et al. [46]	32 cutaneous and oral melanomas	Evaluate the life-long follow-up of dogs affected by cutaneous and oral melanomas treated with episomal CD40L gene therapy.	Twenty out of thirty-two dogs experienced different degrees of side effects, including fever, local swollen lymph nodes, and increased live enzymes. The regular regimen administration adopted is three times a 1 mL injection.
Igase et al. [47]	30 cases with different cancers, including 23 cutaneous oral melanomas	Authors developed different PD-1 monoclonal antibodies (rat–dog chimeric and caninized anti-canine) and evaluated *in vitro* and *in vivo* the efficacy of antibodies.	Nineteen out of 30 patients experienced side effects, including fever and gastrointestinal symptoms. Antitumor responses are evaluated in 24 out of 30 cases and increased overall survival is achieved in vaccinated dogs than in historical control group.
Kamoto et al. [43]	Three oral melanomas	Evaluate a phase I/II clinical trial of an anti-podoplanin monoclonal antibody for canine oral melanoma treatment.	Study demonstrated the monoclonal antibody production and in vivo test in only three animals. No conclusion could be derived based on three subjects.
Maekawa et al. [48]	Nine cases are enrolled with seven having oral melanomas.	Evaluation of the toxicity and safety of a rat–dog chimeric anti-PD-L1 monoclonal antibody for canine cancer treatment.	The vaccine is safe and dogs experienced good antitumor response. However, it is a pilot study with low number of cases.
Piras et al. [45]	42 oral melanomas	Elucidate the disease-free and overall survival times after electro-vaccination with a plasmid encoding human CSPG4.	The protocol is safe and immunogenic, with clinical benefits for the patients.

Reference	Number of subjects and cancer type	Manuscript goal	Manuscript summary
Riccardo et al. [44]	33 oral melanomas	Evaluate the immunogenicity, safety, and therapeutic efficacy of a human CSPG4 DNA-based vaccine.	Authors provided evidence that anti-CSPG4 electro-vaccination prolongs overall survival of dogs with oral melanomas.
Westberg et al. [49]	19 oral and cutaneous melanomas	Evaluate safety and toxicity of a local adenovector CD40L (AdCD40L) immunogene treatment for dogs with oral and cutaneous melanoma.	AdCD40L therapy is safe and may provide benefit to patients.
Grosenbaugh et al. [50]	58 dogs with oral melanoma	Assess the safety and efficacy of a vaccine containing an insert encoding human tyrosinase gene as an adjuvant treatment for canine oral melanomas.	The vaccine is safe and may provide more benefits to the patients as an adjuvant treatment than historical control.

Table 2.
Summary of the articles published in the past ten years with clinical studies evaluating different immunotherapies for canine melanoma.

including intratumoral infiltration of immune cells. The prognostic significance of $CD3^+$ and $CD20^+$ cell infiltration of canine oral melanoma has been investigated; and high infiltration of $CD20^+$ cells is associated with metastasis, frequency of recurrence, shorter survival time, and high rate of tumor-related deaths, and disease-free interval [51]. Therefore, a high infiltration of $CD20^+$ cells is associated with several negative prognostic factors. Yasumaru et al. [52, 53] evaluated the presence of $CD8^+$ and $CD4^+$ infiltrating T cells in oral melanoma using flow cytometry and concluded that tumor-infiltrating lymphocytes predict the aggressiveness and prognosis of patients with oral melanoma.

13. Conclusions

Canine melanoma is a complex disease, usually having poor prognosis when the tumor is located in the oral cavity. Canine melanoma is also a highly immunogenic tumor, with a close association with the immune system. Thus, a better understanding of its immunological components can help in the development of new immunotherapies.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 5

Canine Transmissible Venereal Tumor: An Infectious Neoplasia in Dogs

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Abstract

Canine transmissible venereal tumor is the oldest cancer in dogs and is transplanted via viable cancer cells. This cancer has a specific host, easy transmission, noticeable gross lesions, a predictable growth pattern, an immunologic relative host response, unique molecular characteristics, and is responsive to chemotherapeutic treatment. These points make researchers and practitioners interested in this cancer. Genital cases are noticeable and therefore easier to diagnose and treat than extragenital cases. By contrasting the anatomical features of the two types of cases, we highlight the uniqueness of canine transmissible venereal tumors and discuss the diagnosis, treatment, and prevention of this ancient cancer.

Keywords: canine transmissible venereal tumor, diagnosis, infectious neoplasia, malignancy, treatment

1. Introduction

Canine transmissible venereal tumor (CTVT) is the oldest known contagious cancer in dogs in the world. The first mention of this cancer occurred in the nineteenth century. It is also known as canine infectious sarcoma, canine venereal granuloma, canine transmissible lymphosarcoma, canine round cell sarcoma, and canine Sticker's sarcoma [1, 2]. CTVT has an etiology like that of other contagious cancers, such as devil facial tumor disease (DFTD), which originates from an abnormal cell line with an unlimited proliferative capacity [3, 4]. CTVT can be transplanted via viable cancer cells that naturally allograft between CTVT-infected dogs and uninfected hosts via physical transfer [4].

Previous studies suggest that CTVT cell lineage might be up to 10,000 years old [5]. The clonal origin of CTVT was proven through the analysis of microsatellite polymorphisms, mitochondria DNA (mtDNA), dog leukocyte antigen (DLA) typing, and genome sequencing. It has been suggested by phylogenetic analysis that CTVT emerged between 4000 and 8500 years ago in Asia [6–9]. CTVT is now a common disease worldwide that has been reported on all inhabited continents. However, CTVT has a higher prevalence in tropical and subtropical regions and is uncommon

in North America and Northern and Central Europe, although occasional cases have been reported in imported dogs [5, 10]. An interesting feature of this cancer is that it is usually curable via several protocols. However, metastasis, chemotherapeutic resistance, and death are still reported in CTVT cases in endemic areas, especially in immunosuppressed dogs [1, 11].

2. The carcinogenesis and biological behavior of CTVT

The carcinogenesis of CTVT remains unclear. This cancer may be caused by many sources, but all CTVT cells share the same genetic rearrangement [12]. Specifically, the long interspersed nuclear element-1 (LINE-1) in CTVT cells shows a difference from normal dog cells or host cells [12]. This evidence demonstrates that CTVT clonal evolution grows along the host, and the genetic instability and numerous mutations of CTVT cells give them contagious abilities [6, 8].

Remarkably, CTVT cells are usually transplanted through physical transmission from one dog to another during sexual intercourse. The violent exertions associated with intercourse in both genders are prone to causing genital mucosal damage, which enables the transmission of viable CTVT cells to susceptible hosts. The tumor starts to grow as solitary or multiple nodules at the glans penis or bulbus glandis area in the male dog and on the mucosa wall of the vagina or vulva in the female dog [13]. Cancer cells can affect the mucosa of the external genital organs, skin, and other sites on the body [14–19]. Transplantation occurs individually across the major histocompatibility complex (MHC) between the cancer cells of the CTVT-infected dog and the damaged mucosa of the susceptible dog [1, 2]. CTVT can evade the host's immunological detection, allowing its worldwide spread as a naturally occurring allograft cancer in dogs because its cells lose the expression of MHC class I and II molecules. The growth of a CTVT mass in the external genital area usually appears within 2–6 months after mating [13]. CTVT was the first tumor to be experimentally transplanted by Novinsky in 1876. The CTVT mass cannot be grown with cells that have been treated with glycerin or cell-free filtrates or cells that have been frozen or heated [13]. Even though this contagious cancer has been described as having only dogs as its specific host, CTVT can be heterotransplanted experimentally by inoculation between dogs (*Canis familiaris*) and other members of the social canids, such as wolves (*Canis lupus*), foxes (genus Vulpes), coyotes (*Canis latrans*), and jackals (*Canis aureus*) [1]. The transplantation of viable CTVT cells has also been successful in irradiated mice and athymic nude mice as xenografts in a murine model [13]. Experimentally transplanted and naturally transplanted CTVT growth patterns are predictable and clinically characterized by an initial aggressive growth or progressive phase (P-phase), followed by a stable population in the host or stationary phase (S-phase), and then slowly diminishing cancer cells in a regression phase (R-phase) [20]. In the P-phase, CTVT has a rapid growth rate and forms a mass-like cauliflower feature with discharge at the genital area. Histologic examination of the predictable growth pattern of CTVT in the P-phase reveals numerous round-to-ovoid-shaped CTVT cells with an abundance of mitotic figures and few tumor-infiltering lymphocytes (TILs). The extracellular matrix in the P-phase is rich in the hyaluronan matrix, which may be advantageous for CTVT growth because the hyaluronan creates hydration for the extracellular matrix, which enhances cell proliferation and shields tumor cells against apoptosis [21]. Moreover, the hyaluronan may mask the tumor-associated antigens and MHC antigens on CTVT cell surfaces from the host's immunosurveillance. Histological

features of CTVT tissues in the S-phase show a decrease in the population of CTVT cells; there are fewer mitotic figures and more apoptotic cells than in the P-phase. In the S-phase, the growth rate of cancer cells is slow. Moreover, TILs increase in the S-phase. In the R-phase, the main cellular population is TILs, and the tumor stroma structure gradually collapses and is replaced by collagen tissue. A key feature of the R-phase is the disappearance of cancer cells [22, 23]. Moreover, vascular stroma and fibrosis increase in the R-phase [11, 23]. During the R-phase, the number of myofibroblasts is higher than in the P-phase. This increase in fibroblast population and tenascin-C extracellular matrix coincides with the increasing number of TILs. These features may be the consequence of the same factor produced by the tumor cells and their microenvironment. During the R-phase, the tumor parenchyma destroys and remodels the tumor stroma. Myofibroblasts and extracellular matrices are related to the R-phase and tissue remodeling of CTVT, which are related to wound healing and stromal reactions of tumors [23].

3. Unique features of CTVT and its diagnostic methods

The CTVT mass frequently manifests in the external genitalia of the dog after transmission through coitus. However, other parts of the body can be affected by this cancer. CTVT can be classified into two types according to its anatomical location: lesions typically located on the external genital area of both male and female dogs are called genital TVT (GTVT), while those found in extragenital areas (including subcutaneous, the mucosa of eyes, and in nasal and oral cavities) are called extragenital TVT (ETVT). The GTVT mass at the external genital area is observed as a cauliflower-like mass feature that is friable tissue with hemorrhage or presents with serosanguinous and hemorrhagic discharge and possible secondary bacterial infection [1, 2, 11, 14–18]. ETVT may be related to social behaviors among dogs because of the means of species communication—for example, licking, sniffing, fighting during the breeding season, and routine socialization. As such, the ETVT type is found more frequently in males than in females due to natural behaviors (**Figure 1**) [13].

CTVT has remarkable cytogenetic features. There is an aberration in the number of chromosomes of CTVT. Normally, the normal number of chromosomes in the somatic cells of dogs is 78, 76 acrocentric chromosomes, and couple of metacentric sex chromosomes [7, 21]. Conversely, the number of chromosomes in the CTVT cells varies from about 58–59, with 13–17 metacentric and 42 acrocentric chromosomes and no sex chromosome [1, 7, 13]. These cytogenetic features are consistent and unique and are found in both GTVT and ETVT. This chromosome pattern also appears in CTVT cell cultures and in experimental transplantation [24].

CTVT is one of the round cell tumors, according to its cytomorphologic features. GTVT cases are easier to diagnose according to the location (genital areas) and shape (oozing cauliflower-like mass) of their gross lesions [25]. The CTVT mass can be 0.5 to 10 cm in diameter. Histologic examination of the predictable growth pattern of CTVT reveals numerous round-to-ovoid-shaped cancer cells arranged in a diffuse pattern with an abundance of mitotic figures and few TILs, supported by thin trabeculae of fibrovascular tissue [23]. There are some neutrophils, lymphocytes, macrophages, and plasma cells. The CTVT cell passaging tumor showed no change in the histology of the tumor during the experimental passage [13]. However, the atypical anatomical lesions in ETVT cases are more ambiguous to diagnose based on their location and gross lesion because their features depend on their affecting sites.

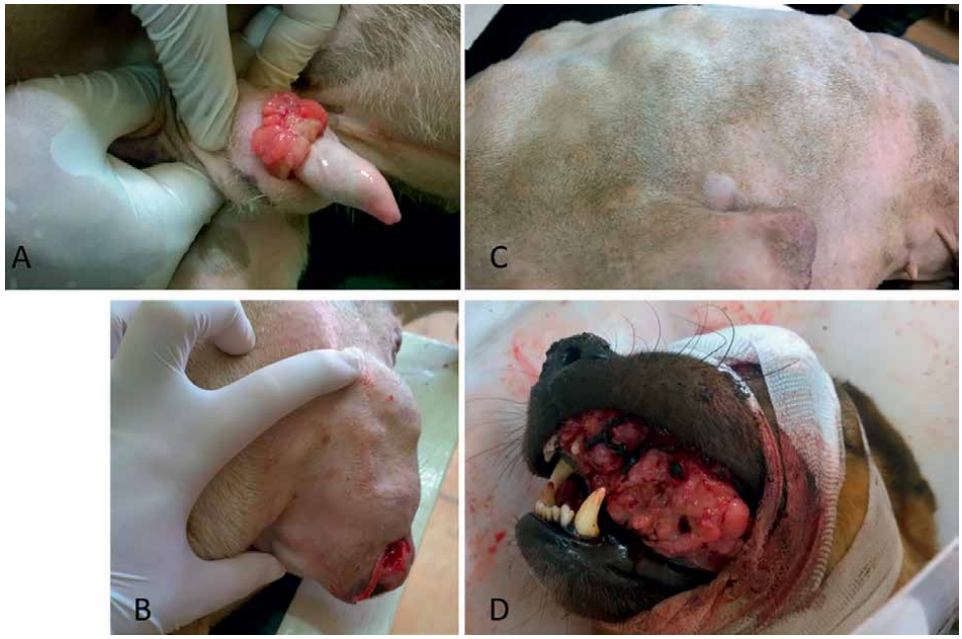

Figure 1.
Gross lesion of genital TVT (1A, 1B) and extragenital TVT (1C, 1D).

For example, they perform like a button mass with ulceration when they are located subcutaneously or on the skin. On the other hand, they display irregular shapes in the conjunctiva and oral and nasal cavities. The ETVT tumor must be differentiated from other types, including mast cell tumor, histiocytic tumor, lymphoma, amelanotic melanoma, and poorly differentiated carcinoma, which depends on the anatomical site of the lesion and the characteristics of the histologic examination [25].

Cytologic diagnosis is of great value for easy and rapid on-site diagnosis [26]. As mentioned before, CTVT is one of the round cell tumors, so the main populations of CTVT cells are round-to-ovoid-shaped cells that may originate from the histiocytic system. To improve cytologic knowledge, researchers found that there are three types of cytomorphologic classification of CTVT, which are categorized by the cell morphology of the majority population: (1) lymphocytic type, characterized by more than 60% of round cells, with fine granular cytoplasm, central nuclei, and few intracytoplasmic vacuoles; (2) plasmacytic type, characterized by containing more than 60% of cells with broad cytoplasm, eccentric nuclei, and large amount of vacuoles; and (3) mixed type, presenting both lymphocytoid and plasmacytoid cells, neither of which exceeds 59% [26, 27]. Recently, a study with computerized cytomorphometric analysis of round cells revealed that CTVT had the largest cellular and nuclear size which followed by the histiocytic tumor cell, mast cell tumor, and lymphoma cell. CTVT cell from GTVT case had the largest cellular and nuclear size followed by CTVT cell from ETVT case, histiocytic tumor cell, mast cell tumor cell, and lymphoma cell. According to the CTVT cytomorphologic type, the mixed type had the largest cellular and nuclear size followed by the plasmacytic and lymphocytic type. The researchers have revealed that the plasmacytic type is the most common cytomorphologic type [27, 28]. The plasmacytic type [26–30] and the mixed type [26] are related to malignant behaviors and chemotherapeutic drug resistance.

The lymphocytic type shows aggressive behavior less than other types [26]. So, cytomorphologic classification can provide a prognostic for treatment in each CTVT case (**Figure 2**).

Most canine round cell tumors have been immunocharacterized using several tumor markers—for example, cluster of differentiation 3 (CD3), CD79, paired box-5 protein (PAX-5), and protein-tyrosine kinase (c-kit and CD117). Diagnosis and classification using the immunophenotype are more accurate than routine histopathologic examination. However, the cell origin of CTVT is unclear. CTVT has been previously described as lymphosarcoma, a round cell sarcoma, a histiocytoma, and a tumor of neuroectodermal or reticuloendothelial origin [23]. Immunohistochemistry studies revealed that CTVT cells are negative for keratins, α-smooth muscle actin, desmin, CD3, immunoglobulins G and M, γ-light chains, and κ-light chains. These panels ruled out epithelial, smooth muscle, and T- and B-lymphocytes. CTVT cells are positive for vimentin, ACM1, lysozyme, and alpha-antitrypsin (AAT). Lysozyme and alpha-antitrypsin are not expressed by other mesenchymal cells. Moreover, ACM1 is a canine-specific antibody recognized in canine mononuclear phagocyte stem cells. This panel immunophenotypic expression suggests that CTVT has a histiocytic origin because this set of antigens is not expressed by other mesenchymal round cells [23].

CTVT has unique molecular characteristics. CTVT has the rearrangement of the c-*myc* gene, which is related to the LINE-1 [12, 31]. The LINE-1 is a retrotransposon or jumping gene localized at the 5′ region to the exon of the c-*myc* locus of CTVT cells. This jumping gene causes destabilization in the entire cellular genome, leads to cellular proliferation and differentiation, and performs the malignant transformation of cells. A rearrangement of the LINE-1–c-*myc* gene sequence has been used with polymerase chain reaction (PCR) and *in situ* PCR to diagnose CTVT [6]. The localization of the LINE-1 positive was in the nuclei of CTVT cells; this was not present in other inflammatory cells or CTVT connective tissue. The LINE-1 is inserted in all cases in the same position and presented in the same PCR product size. This method can be performed using fine-needle aspiration (FNA) samples with only 10 nanograms of

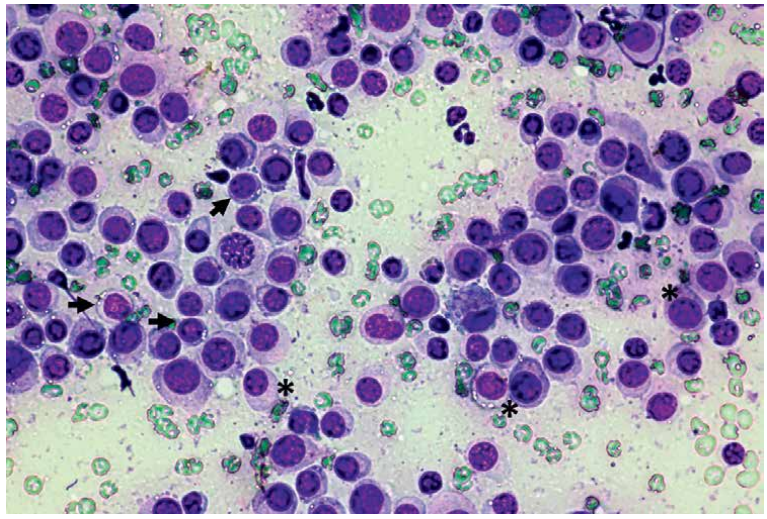

Figure 2.
Cytomorphology of CTVT cells presents the lymphocytic cell type (arrow) and plasmacytic cell type (star); H&E (40X).

sample DNA for conventional PCR. The LINE-1–c-*myc* PCR revealed a diagnostic sensitivity of 100% and specificity greater than 80% [25]. As mentioned before, the CTVT somatic cell lineage is remarkably stable and lacks subclonal heterogeneity despite many genome rearrangements, copy number changes, and retrotransposon insertion [8]. The unique, specific, and constant molecular feature of CTVT is utilized as a definitive diagnostic marker and for applicability in veterinary clinical routines because this feature is found in normal CTVT and vincristine-resistant CTVT cells. Also, the PCR method using FNA samples can be used as a surveillance protocol during and after chemotherapy to determine complete remission status and the appropriate times to stop or restart chemotherapy [11].

4. Treatment of CTVT

CTVT cells respond to many forms of therapy. Historically, CTVT has been treated with surgical excision therapy and followed by several chemotherapeutic cocktails or a combination of chemotherapeutic protocols or radiotherapy. Surgery is chosen for a localized CTVT mass, but it is not recommended in generalized or metastatic cases. Electrosurgery and cryosurgery are optional surgical methods because the tumor is easily transplanted to surgical wounds when using the conventional surgical method. The rate of recurrence after conventional surgery was between 12 and 68% [13]. Surgical methods have a higher recurrence rate than chemotherapy. Radiotherapy was chosen as the therapy for CTVT cases with complete regression within 1–3 treatments of a dose of 10 Gy or 1000 rad. Although radiotherapy is a successful treatment, this method requires sedation for dogs, specialized technicians, and expensive equipment [13].

The original combining protocol was composed of vincristine, cyclophosphamide, and methotrexate. Also, there have been attempted combination protocols, such as cyclophosphamide and prednisolone, vinblastine with cyclophosphamide, vinblastine with methotrexate, and vincristine with ivermectin [13]. After clinical evaluation and development of chemotherapy for CTVT treatment, the use of vincristine, vinblastine, and doxorubicin, as single agents were attempted. The duration of vincristine for complete remission is around 4–6 weeks of intravenous administration at a weekly dose of 0.025 mg/kg bodyweight [11, 32]. CTVT cases are treated using vinblastine intravenously in a dose of 0.1 mg/kg bodyweight on four to six weekly treatments [13]. Moreover, complete remission was also reported within three weeks in the doxorubicin treatment when given intravenously at a weekly dose of 30 mg/m^2 surface area [19, 32]. Vincristine has been reported as the most effective, safe, and convenient agent for GTVT and ETVT cases. The better response to vincristine treatment may be due to less myelosuppression compared to doxorubicin and no immunosuppression by methotrexate. However, weight loss, mild leukopenia, and gastrointestinal toxicity, such as anorexia, nausea, and vomiting, are common adverse effects of vincristine treatment in less than 10% of cases. An additional complication of vincristine treatment is the extravasation of the drug during administration causing necrotic ulcer lesions of the affected skin [19].

Recently, the combination of vincristine (VCR) and L-asparaginase (LAP) protocol or VCR-LAP protocol has demonstrated an effective treatment result and shorter treatment time than VCR alone. The VCR-LAP combination protocol is given

in alternating weekly doses of 5000 IU/m^2 of LAP subcutaneously and 0.025 mg/kg of VCR intravenously. The duration of combination protocols is 2–5 weeks, with no evidence of VCR-resistant cases. The short period of treatment provides fewer opportunities for chemotherapeutic drug resistance. Moreover, the combined protocol costs less on average than mono-chemotherapy [11]. A comparative treatment study via a murine model of vascular targeted photodynamic therapy was performed as the new strategy for chemotherapeutic-resistant cases [33]. Moreover, VCR-resistant status is still increasing, not only in ETVT cases but also in GTVT cases [11, 32, 34]. When only partial remission was noted in seven and eight weeks, a 30 mg/m^2 dose of doxorubicin was administered as mono-chemotherapy every three weeks for a total of five treatments [19]. The duration of doxorubicin treatment was around three applications or two months. In addition, four treatments of vincristine 0.025 mg/kg bodyweight with LAP 10,000 IU/m^2 every two weeks were also used in resistance cases that showed complete regression [34].

The best thing for veterinarians to keep in mind is that this contagious cancer can be treated with chemotherapy and achieve complete regression. Also, the mono-chemotherapeutic drug VCR can cure this cancer and is recommended as the chemotherapeutic drug of choice for CTVT treatment. Recovery rates are high in more than 90% of cases and have been documented by using VCR at a 0.025 mg/kg body weight dosage over two to eight weeks. The mixed and plasmacytic cytomorphologic types show malignant behavior related to vincristine-resistant and recurrent cases [26]. However, the lymphocytic type has been shown to be less malignant than other types. The larger cell size or the increase in the cellular and nuclear size of tumor cells may demonstrate the survival ability of cells and the progression of tumor grading [35, 36]. Also, the lymphocytic type was found in GTVT cases and was not related to metastasis behavior [26]. According to the anatomical lesion, this can infer that GTVT has a lower malignant behavior than ETVT [11, 26]. In VCR treatment cases, the lymphocytic type had the shortest time to complete regression. The prognosis of treatment with VCR is influenced by the stage of growth, the cytomorphologic type, the size of the tumor, the anatomical site of the mass, and the climate [13, 26, 32]. Recurrence of CTVT was rare because of the effectiveness of chemotherapy. However, some recurrent cases were reported six months after complete remission. So, long-term monitoring after cessation of treatment should be more than six months (**Figure 3**) [11, 32].

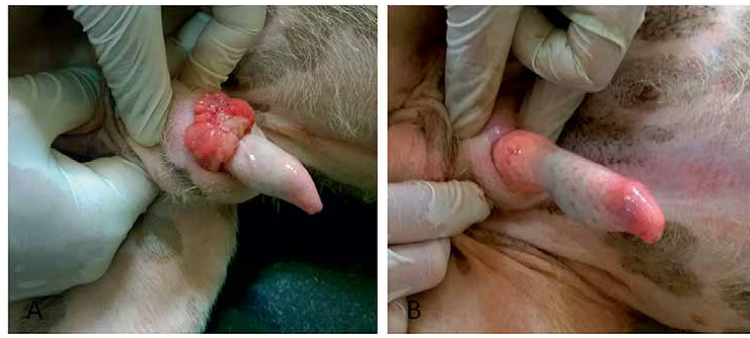

Figure 3.
The lesion at the penis before (3A) and after complete remission (3B).

5. Immunologic relative host response and factors influencing the susceptibility of dogs to CTVT

The CTVT mass grows mostly on male and female external genital areas due to live cell transmission during coitus. The highest risk periods are the estrus and breeding periods. No breed predisposition has been documented, but mixed breed dogs were reported in 41% [19] to 100% [11] of cases. Dogs of any age are susceptible to CTVT. CTVT is most commonly found in intact dogs between two and five years of age. The mean ages of the affected dogs were 3.9–4.5 [13]. GTVT is never found in virgin dogs. Also, the tumor is more common in females than males due to one infected male often interacting with many females, both in breeding kennels and endemic areas [13]. Older dogs showed more ETVT evidence than GTVT, which is more common in intact young adult dogs. Poor body condition scores and immune status might be cofactors in aging dogs with ETVT [11].

Researchers have been interested in and attended the roles of host immunity response in the P-phase and R-phase of CTVT. In experimental transplantation, CTVT can evade immune surveillance and show rapid growth for 12 weeks. The spontaneous regression of both natural and experimental transplantation suggests that the host immune response plays a major role in CTVT. Moreover, immunosuppressed dogs and puppies develop more aggressive CTVT masses that lack TILs, and these masses are rarely eliminated and hardly show complete remission [2]. The differences between the P-phase and the R-phase are the presence and number of TILs. Thus, the complete regression and complete response to treatment may depend on the appropriate immune response of host cells, which is related to the immune status of the CTVT-susceptible dogs. CTVT cells evade immune detection during the transmission period and growth phase by secretion of transforming growth factor β (TGF-β). TGF-β is a multifunctional protein that controls cellular differentiation and proliferation, which acts as a suppression activity of class I and II MHC expression and natural killer (NK) cell activity. Conversely, TGF-β is countered by interleukin-6 (IL-6), which is produced by TILs [20, 37].

CTVT cells show the tumor-associated antigen and shed into the circulation during the P-phase, and this CTVT tumor-associated antigen could no longer be detected after surgical removal of the CTVT mass [13]. This CTVT tumor-associated antigen was detected using antibodies against the antigen during the P, S, and R phases [38]. The amount of this antigen released in the host circulation increased alongside the increase in tumor volume. There is evidence that humoral immunity plays a role in the P-phase. This antigen may be responsible for blocking the host's immune response in the P-phase. The infiltration, the presence of B cells, and the upregulated expression of the groups of genes related to B cells were mentioned in the signature of acute allograft rejection [13]. Thus, humoral immunity also plays an important role in inhibiting and preventing CTVT tumor development and acute CTVT allograft rejection in many case studies. This emphasizes that CTVT itself has been reported to be antigenic and can evoke tumor rejection in the host's immunity.

In naturally occurring CTVT, spontaneous regression was also observed, albeit less frequently than in the experimental transplanted CTVT [32]. The dogs that have recovered are immune to reinoculation [1]. Passive immune transfer of post-regression sera to CTVT-bearing dogs has been shown to inhibit and prevent tumor development. Moreover, the growth of CTVT is inhibited in puppies born to dams that are immunized for CTVT before or during pregnancy [2]. CTVT cells have more membrane-bound antibodies in the S and R phases than in the P-phase.

The absence of antibodies in puppies with metastasis suggest the importance of humoral immunity in progression. The metastatic rate is low—less than 20% in GTVT cases [9, 19]. Metastatic cases have been reported in subcutaneous tissues, skin, lymph nodes, eyes, tonsils, liver, spleen, oral mucosa, nasal mucosa, brain, and bone marrow [15, 21, 32, 39]. Although metastasis is not common in CTVT cases, it can be a serious situation and the cause of death.

6. Immunotherapy for CTVT treatment

Recently, researchers have found that inflammation and epithelial cell proliferation may characterize the early response to VCR treatment in the early stage [40]. CTVT in the R-phase after treatment showed that the expression of many groups of genes occurred at the same time, with pathological changes of not only macroscopic features but also microscopic ones. The group of inflammation genes was the most upregulated in the S-phase, and the immunologic groups of genes involved in T-cell, NK-cell, and B-cell function were upregulated in the R-phase. In this late R-phase, there was a loss of CTVT cells and cell migration but an increase in fibrosis that is related to new tissue formation or the healing stage [23, 40]. This finding revealed the process that started with the inflammatory response, epithelial and keratinocyte proliferation and followed by the host T-, NK- and B-cell infiltration, and finished with the cell cycle arrest. In addition, Bcell-related genes, albeit less prominent in quality and expression levels than T- and NK-cell panels, were also progressively upregulated [40]. This is related to previous studies that showed that the infiltration and presence of B-cell was the signature of acute allograft rejection [41].

The interferon (IFN) for neoplasia treatment is initially based on the non-specific activating host immune response. Normally, type I IFN has the ability to inhibit tumor cell growth and induce tumor cell apoptosis in the in vitro studies [42]. During the R-phase of CTVT growth, the IL-6 and interferon-γ or Type II IFN from the host plays a special role in enhancing MHC molecule expression on antigen-presenting cells, activating NK-cell activities, and modulating B lymphocyte responses [20, 37, 43]. An in vitro study of interferon type I for CTVT treatment found that interferon ω showed the effect of inhibiting CTVT cell viability in a dose-dependent manner [44]. CTVT case treatment by immunotherapy is of interest to many researchers. Recently, the combination protocol of intratumoral interferon-α2a with VCR shortened the treatment duration when compared with VCR alone [45]. Thus, combining a low-dose chemotherapeutic drug and immunotherapy may be advantageous for CTVT patients because of the initial trigger of inflammation by chemotherapy, synergizes with the activation of the host immune response by interferon. The VCR triggers host interferon signal expression, which induce NK-cell and lymphocyte infiltration. The addition of interferon may enhance the innate and adaptive responses of mononuclear cells and might affect CTVT viability and proliferation. The change in environment and the increase in inflammatory production by local host cells after treatment with VCR may trigger and recruit immune cells. The strong response induced by VCR causes the release of damage-associated molecular patterns from stressed or apoptotic cells as an innate immune response of the host, which induces direct cognition of foreign DLA molecules and ultimately leads CTVT to regression. In addition, this evidence suggests that combining the low dose of chemotherapy with immune checkpoint therapy may help the host immune response against CTVT by inducing the inflammation for tumor regression (**Figure 4**) [40].

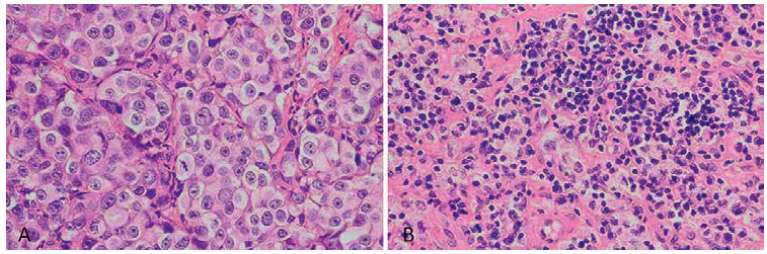

Figure 4.
Histopathologic features of CTVT tissue at week 0 or before treatment with vincristine (4A) and two weeks after treatment (4B).

7. The ecology, control, and prevention of CTVT

Stray dogs and poor policy control are the predisposed causes of CTVT transmission [19]. Thus, the control of CTVT transmission is difficult because free-roaming dogs and their intact status represent a reservoir [9, 13]. Prevention is related to government policy, maintenance of spay and neuter campaigns, and animal feeding practices in each country [9]. CTVT cases are found more often in rural areas than in urban areas because of a lack of adequate veterinary services [13]. Currently, CTVT is estimated to be found at a prevalence of 1–10% or more in dogs in many countries on all inhabited continents. CTVT is endemic in at least 90 countries worldwide. The highest prevalence of CTVT was recorded in Belize, where the prevalence was 10–20%. However, prevalence is decreasing in North America and central and northern Europe [9]. New owners and breeders should conduct a careful physical examination before adoption or breeding, especially in imported dogs. Dog licensing laws, spay and neuter encouragement campaigns, and controlling stray or free-ranging dogs should be emphasized to reduce physical contact between infected and uninfected dogs. Also, long-term monitoring of 6–12 months after cessation of treatment should be performed and this practice should be encouraged among veterinarians in endemic areas.

8. Conclusion

CTVT is the only naturally occurring contagious cancer in dogs. This oldest canine cancer spreads through the physical transfer of whole viable cancer cells between hosts. The specific host, transmission, gross lesion, microscopic features, growth pattern, immunologic relative host response, molecular characteristics, and responsiveness to treatment of CTVT are of interest to researchers and practitioners. Genital CTVT cases are visually noticeable and are easier to diagnose and treat than extragenital CTVT cases. The conventional single chemotherapeutic agent VCR has delivered curable treatment in most CTVT cases during 4–6 chemotherapeutic cycles. However, vincristine-resistant cases have been increasing in number. This decade has revealed more treatment options, such as VCR–LAP combination protocol. By contrasting the anatomical features of the two types of cases and the VCR-resistant cases, this paper highlights that the GTVT type is more noticeable and curable than the ETVT type.

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Conflict of interest

The authors declare no conflict of interest.

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Section 3

Animal Disease Solutions

Chapter 6

Diseases of the Canine Prostate Gland

Sabine Schäfer-Somi

Abstract

In dogs, the most frequent diseases of the prostate gland are benign prostate gland hyperplasia (BPH), acute and chronic prostatitis, squamous metaplasia, and prostate tumors. New diagnostic tools comprise diagnostic markers in the blood and urine, as well as advanced imaging methods. The therapy can be initialized with the 5α-reductase-inhibitor finasteride or an anti-androgenic compound, and prolonged with a long-acting gonadotropin-releasing-hormone (GnRH)-agonist such as deslorelin. In case of prostatitis, effective antibiotics must be applied for weeks. Antibiotics must be able to penetrate into the prostate tissue; fluoroquinolones, clindamycin, and erythromycin are good choices and are in addition effective against mycoplasms. The chronical prostatitis cannot be differentiated from a neoplasia by sonography; a biopsy, histological, and bacteriological examination are required. Tumors of the prostate gland are seldom and mostly occur in castrated but in intact dogs. For the final diagnosis, a biopsy must be taken. Partial and total resection of the prostate gland by use of laser technique is possible but coincedes with many side effects and the prognosis is still futile. Immunotherapy combined with NSAIDs, targeted noninvasive thermotherapy, *BRAF* gene inhibitors, or prostate artery chemoembolization are promising methods.

Keywords: dog, prostate gland, BPH, prostatitis, tumor

1. Introduction

The prostate gland is the only accessory sexual gland in the male dog. Some authors in addition name the ampullae ductus deferentes. The canine prostate secretion is a transport medium among others for passive transportation of the spermatozoa into the uterus during ejaculation. The prostate secretions furthermore influence the motility and function of the semen cells; the exact composition and many functions are not yet known and vary dependent on the laboratory and the analysis method used [1]. The composition of mineral nutrients, as well as the amount of cholesterol, albumin [2], zinc-binding proteins [3], fertility-associated proteins like osteopontin [4], the antioxidative capacity [5], and many more have been examined. A new study investigated the composition of the seminal plasma by use of mass-spectrometry [6].

Diseases of the prostate gland frequently occur in aging dogs [7, 8]; the incidence increases with age: 6.2% in intact males with ≤ 4 years, 17.5% in 4–7 years old dogs,

32.8% in 7–10-years old dogs, and 43.5% in male dogs >10 years of age [9, 10]. Diseases of the prostate gland can be infectious or noninfectious. In aging dogs, the noninfectious benign prostate gland hyperplasia (BPH) is the most frequent disease, occurring in 80% of all intact male dogs older than 5 years and in >95% of male dogs older than 9 years [11, 12]. The BPH can be easily treated; however, the disease will become chronical with regular rezidives and only castration will finally cure the dog. Inflammatory diseases may be chronical or acute; the acute prostatitis is mostly caused by bacterial infections, either ascending via the urethra or via the bloodstream. The chronical prostatitis develops from a BPH or an acute prostatitis if treated with wrong antibiotics or for a too short period of time [7]. Highly effective antibiotics applicated for a sufficient period are essential for the successful treatment of the prostatitis.

The squamous metaplasia develops due to hyperestrogenism occurring because of endocrine testicular tumors; however, may also be caused by estrogen applications [8].

Prostate tumors are relatively rare in dogs, the incidence is on average 0.43% [13], they mainly occur in older dogs and more frequently in castrated than in intact dogs; the growth is not androgen-dependent [14–16]. In this chapter, modern diagnostics and therapeutical methods are discussed.

The aim of each treatment must be, to hinder the development of chronical diseases, for prevention of the long-term use of antibiotics that are needed for special infections [17]. Regular examinations, best starting when the dog reached 40% of life expectancy, will help to reach this goal [18].

This article provides an overview of diagnostical and therapeutical measures in different prostate gland diseases and insights into at present most actual developments.

2. Anatomy of the prostate gland

The canine prostate gland consists of two parts, surrounding the caudal part of the urethra; it is round to oval and has a sulcus dorsal and ventral that can be reached by digital rectal palpation. It is surrounded by a thick fibro-muscular capsule releasing septs of smooth muscle tissue into the gland. The urethra is situated in the middle of the prostate gland and between the two parts. The situ of the gland is dependent on age; in young dogs, it is situated in the pelvis, in aging dogs more in the abdomen, and because of an increasing size of the diseased gland, in the old dog, it can be situated in the pelvis again. In this case, it can be examined by digital-rectal palpation again. The cranio-dorsal and cranio-ventral part of the gland is covered by peritoneum. The glandular ducts open into the urethra at the site of the pars disseminata and on the colliculus seminalis. The blood supply is provided via the arteria pudenda interna, innervation by the hypogastric nerve [19].

3. Function/endocrine regulation of the prostate gland

The prostate reaches maximum secretory activity in dogs of on average four years of age [20], the secretions comprise >90% of the ejaculate; the gland continues to grow under the influence of testosterone because of stem cell differentiation, and in the aging dog will increase in size because of hypertrophy and hyperplasia. Growth and secretion are regulated by the active metabolite of testosterone (T), namely the

5α-Dihydrotestosterone (DHT). More than 95% of testosterone are converted into DHT by the enzyme 5α-reductase, after diffusion into the prostate gland cells. DHT binds stronger to the testosterone receptor than T [21, 22]. Estradiol-17ß supports the effect of DHT in a synergistic way and, in addition, causes an upregulation of testosterone receptors [11].

The prostate gland secretion supports the transport and the function of spermatozoa after ejaculation. It contains citrate, lactate, cholesterol, and enzymes; however, few sugars and phospholipids are supposed to provide additional energy. The composition of the prostate secretions was recently investigated by means of proteomics [6, 23]. The serine-protease canine prostate specific esterase (CPSE) and the lactotransferrin-precursor are the most frequently occurring proteins in the seminal plasma [23], comprising 90% of all proteins. The CPSE has a proteolytic effect, similar to chymotrypsin [24] and after binding influences spermatozoa function by its zinc-binding properties [3]. The CPSE binds to phosphorylcholine-binding protein and choline phospholipid of the membrane and induces the efflux of cholesterol from the spermatozoa membrane during ejaculation, which is essential for capacitation. The secretion of CPSE is controlled by androgens [25] and the enzyme is believed to be a reliable marker of prostate secretion [26].

The extracellular matrix of the canine prostate (noncellular stroma and fibrous tissue) supports the development of the gland and the control of cellular functions [21], supposedly via cellular transmitters like cytokines [27].

4. Diseases

4.1 Benigne Prostate Gland Hyperplasia (BPH)

This noninfectious disease of the prostate gland only occurs in intact male dogs with endocrinally active testicles. The disease counts for 50% of all prostate diseases [9]. The incidence increases with increasing age; however, in rare cases, BPH can occur at the age of 2–3 years [28]. Sonographical and in part clinical symptoms usually can be seen in 80% of male dogs at the age of 5 years [11, 12, 20], and in >95% of males at the age of >9 years [11]. The hyperplastic increase in size is caused by:

- A change in steroid-hormone-concentrations
- An increasing estrogen: testosterone ratio in the intact, aging dog [29]
- A change in the receptor expression within the gland and especially by increasing concentrations of DHT in the epithelial, hyperplastic tissue.

Dihydrotestosterone is the active form of testosterone and produced from testosterone by the enzyme 5α-reductase. The activity of the enzyme increases in the aging dog, especially in the glandular epithelial cells; therefore, the hyperplasia mainly concerns the glandular epithel and less the stroma [30, 31]. In one experiment, BPH could be produced by long-term application of 5α-androstan-3aα, 17ß-Diol (3α-Diol), in combination with 17ß-Estradiol. 3α-Diol is produced by reduction from DHT and/or 17ß reduction from androsterone; it stimulates the intracellular cAMP production in the prostate gland [32]. In another experiment, the testosterone concentration was doubled on days 21 and 42, with the same effect [33]. The experiment points toward

the impact of these hormones and an eventual change in the enzyme and metabolic activity inside the aging gland. The role of local growth factors and relaxin is still not sufficiently investigated.

Prolactin was detected in prostate secretions of dogs with BPH and with higher concentrations than in healthy dogs; during the development of the prostate, prolactin contributes to growth and differentiation [29].

As a further predisposing factor, the breed was previously mentioned; large breeds seem to be more often concerned [34–36] and in a recent study, the Rhodesian Ridgeback was shown to be predisposed, pointing toward a genetic cause [35]. Some authors suggest a breed-specific pituitary prolactin secretion, which lacks evidence so far but deserves better investigation [35, 37].

4.1.1 Clinical symptoms

The disease starts with centrifugal increase in size; sonographically, changes in echogenicity and cystic caverns become visible. Clinical symptoms develop later on [18]. Therefore, the BPH can be termed a physiological process in aging dogs, until clinical symptoms occur (Tsutsu et al. 2000).

The first clinical sign mostly is serosanguinous preputial discharge not associated with urination; this discharge occurs because of vessel damages in the hyperplastic, well-perfused tissue [38].

The secretions reach the urinary bladder via the pars disseminata causing a bloody admixture of the urine [9, 38]. In breeding dogs, a changed composition of the prostate secretions causes an increase in pH, a decrease in motility, and bloody prostate secretions [39, 40]. Later on, morphological aberrations of spermatozoa occur [19]. BPH may cause reversible infertility. Abdominal pain because of the enlarged gland is seldom [9, 19]. The centrifugal growth of the gland causes compression of the urethra and can cause dysuria, dyschezia, stranguria, and even anuria; however, the latter is seldom [11, 41], and urination problems were seen in only 27% of dogs with BPH in one study [9]. Defecation problems more frequently occur, especially in advanced stages of BPH due to compression of the rectum, leading to acute constipation in extreme cases [19, 42].

4.1.2 Diagnosis

For an accurate diagnosis, a case history, a clinical-andrological examination of the dog including digital rectal palpation and abdominal sonography are obligatory. Furthermore, examination of urine and semen, as well as cytological examination of the prostate gland secretions can be helpful. Zambelli et al. [43] used the parameters anorexia, loss of weight, degree of tenesmus and dysuria, urinary incontinence, preputial discharge, and hematuria for clinical grading of the BPH in 4 grades, with grade 1 corresponding to asymptomatic BPH.

Digital-rectal examination reveals a symmetric increase in size, normal consistency, and no painfulness; large intraprostatic cysts may cause asymmetry [38].

Sonography is a good method for diagnosis of BPH in dogs; however, it should always be combined with further clinical methods [44]. The quality of the examination is variable and dependent on the quality of the pictures, and the reproducibility of the measurements [45], as well as the position of the probe [9]. Sonographical parameters are size, structure, echogenicity, and abnormal structures such as cysts, abszesses, mineralization, asymmetry of the lobi, etc [18, 46] (**Figure 1**).

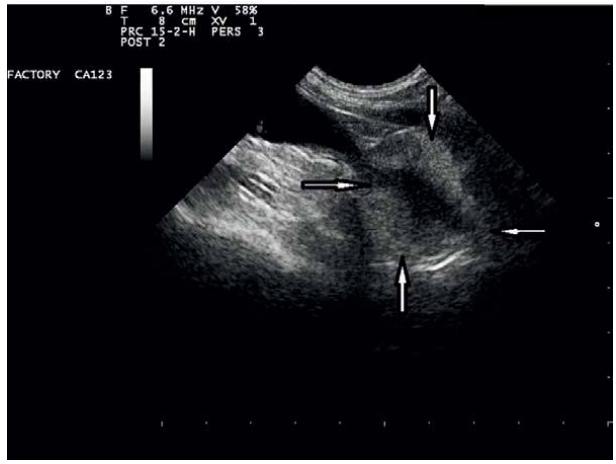

Figure 1.
Sonography (B-mode) of the physiological prostate. Prostate gland of a healthy 5-year-old beagle, the white arrows mark the contour. The size was 3 × 2 cm (L × W), the structure of the gland is homogenous, the echogenicity is physiological, and the anechoic line in the middle is the urethra.

The volume of the gland correlates with the body weight [47] and can be calculated when length (L), width (W), and height (H) were measured by using a formula; for example,

Measured L × W × H × 0.523/estimated volume (0.33 × body weight in kg × 3.28).

In dogs with BPH, this ratio will be > 2.5 [48].

4.1.2.1 Sonography

With B-mode sonography, the prostate with BPH appears enlarged, and the parenchyma is homogenous and hyperechogenic. Intraprostatic cysts of different sizes are frequent (Abb.3), and paraprostatic cysts sometimes occur [8, 12, 18, 45] (**Figure 2**). Cysts are round, thin-walled structures with anechoic contents and distal increases in echogenicity [45].

When using special doppler-sonographical methods like power or pulse-wave Doppler sonography in dogs, the examined vessels [49], as well as previous ejaculations and medications, have to be considered; a sexual rest before the examination is recommended [50]. The case history should reveal whether a gonadotropin-releasing-hormone(GnRH)-analogon was applicated previously, which will change the findings considerably [51].

An increase in perfusion of the gland was recorded in 8/16 dogs with BPH in one study, using pulse-wave Doppler sonography [46]. In another study, peak-systolic velocity (PSV) and end-diastolic velocity (EDV) were significantly higher in dogs with BPH than in healthy controls [52].

Contrast-enhanced sonography (CEUS) proved to be advantageous for evaluation of vascularization and perfusion of the canine prostate gland. For this method, ultrasoundcontrast agents (UCA) are injected intravenously. Unfortunately, the use of different UCA makes results from different studies difficult to compare [45]. In one study, healthy male dogs were injected with a micro-bubble UCA with the aim to obtain physiological reference values [53]. However, one study is not sufficient; the generation of reference values by using a large and comparable data pool, standardized methods, and settings is a big problem.

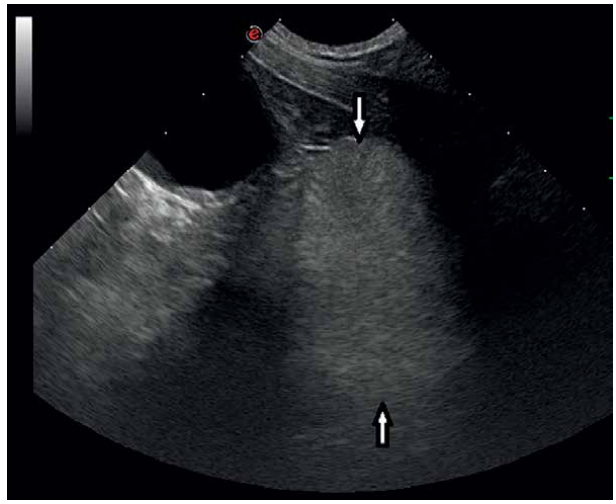

Figure 2.
Sonography (B-mode) of a prostate gland with BPH. The prostate is high-grade enlarged (arrows), the structure is homogenous, and the echogenicity is increased. The dog showed bloody preputial discharge, stranguria, and defecation problems.

In an earlier study, micro-bubble UCA and CEUS proved to be useful for detection of vessel damages and necrosis. Unfortunately, it is still not possible to differentiate between BPH and chronical and acute prostatitis, respectively [54]. However, together with further diagnostic methods, these sonographical tools provide worthful diagnostic findings.

Elastography is an interesting tool for evaluation of tissue consistency, the degree of elasticity, and rigidity. The principle is that the degree of deformation after pressure on a certain tissue is inversely proportional to the rigidity of this tissue [55]. Different methods such as acoustic radiation force impulse elastography (ARFI) [56] were evaluated in dogs. With qualitative ARFI, short acoustic impulses of high intensity are used for deformation of the tissue, then the data are converted into a statistic grey scale (Elastogram), revealing the rigidity of the examined tissue. With quantitative ARFI, an acoustic wave is sent in a certain region of the tissue, spreading at a certain velocity within this tissue, and dependent on the rigidity of the tissue. The measured velocity correlates to rigidity and viscoelasticity of the tissue [57]. For examination of the canine prostate gland with elastography, physiological values for different groups of age are available [56, 58, 59]. Unfortunately, no controlled study about the use in dogs with BPH is available. The method requires some training.

Echostructure analysis or computerized histogram analysis of sonographical pictures is a method well-known in human medicine for diagnosis of mammary tumors. Similarly in dogs, the method proved to be useful for the diagnosis of mammary carcinomas [60]. For this method, the gland is examined via B-mode sonography and the pictures are digitalized. Then so-called regions of Interest (ROI) are marked in the pictures (**Figure 3**) and objectively evaluated by using computer-assisted analysis. (software: for example ImageJ; Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA) The echostructure analysis provides information about brightness, micro- and macrotexture, homogeneity, and contrast differences within a certain tissue [60, 61]. In a previous study, the echostructure method was used to

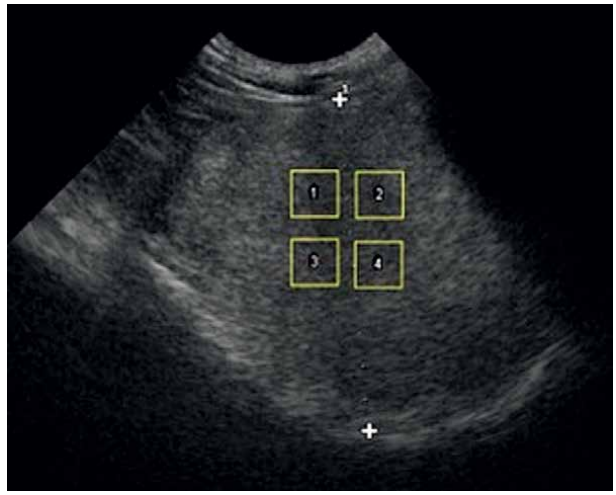

Figure 3.
Echostructure Analysis of the prostate gland, regions of interest (ROI). For the objective analysis of the digitalized, B-mode pictures, four quadrants of equal size have to be placed in the region of interest. The measures are performed automatically in these regions. Evaluation is performed by a special software.

differentiate between BPH and chronical prostatitis. In dogs with BPH, the homogeneity of the gland tissue was significantly higher than in the dogs with chronical prostatitis [62].

4.1.2.2 X-ray

X-ray of the canine prostate gland provides information about the size and situ of the gland. In healthy dogs, the diameter of the prostate is at a maximum 70% of the distance between the cranial margin of the pubic bone and the promontory of sacrum [63], an increase in size points toward BPH. By using a retrograde urethrocystogram, examination of the urethra is possible; the lumen can be confined by BPH, abscesses, or neoplasms [63]. In a previous study, power injection of a contrast medium during retrograde CT-urethrography improved the evaluation of the urethra; dilations of the urethra could be easier evaluated in relation to the degree of the filling of the urinary bladder [64].

Diseases of the prostate gland can be diagnosed by use of *computer tomography* (CT); data about the healthy canine prostate gland are available [65]. The appearance is round to ovoid, homogenous, and well definable, whereas capsule, stroma, and parenchyma cannot be well differentiated. In dogs with BPH, prostate megaly, decreased density, and heterogeneity of the tissue are characteristic; reference values for the size are available [66]. However, in the cited study, the groups were heterogeneous and the size of the dogs variable. The use of a contrast medium facilitates the diagnosis, especially evaluation of the median septum and the vascular system [65, 67]. In one study using contrast-CT, the results correlated well with the cytological findings [68]. An important advantage of CT is the possibility to recognize and localize metastases [66]. However, since the CT examinations have to be done under general anesthesia, examinations should only be done in suspected cases of prostatitis, carcinoma, or other masses.

A rather new method is the diffusion-weighted and perfusion-weighted *Magnet resonance Imaging* (MRI). In a recent study, the prostate gland of healthy beagles

was examined and physiological values for perfusion and diffusion were obtained [69]. electrical-conductivity-based MRI is a further method, helping to recognize changes in the canine prostate gland tissue by evaluating changes in the contrast [70]. Further MRI methods are available and will be discussed in the chapter about prostate carcinomas [71].

4.1.2.3 Examination of blood, urine, seminal plasma, and the sperm-rich phase

In case of canine BPH, *examination of blood* parameters usually reveals normal findings, whereas *examination of the urine* in some cases reveals hematuria [34, 38]. *Seminal plasma* and *prostate secretions* often contain erythrocytes [40], *spermatozoa* often show a decrease in motility and an increase in morphological abnormalities [19, 39, 40].

A *bacteriological examination* of prostate secretion, urine, and semen is often negative and may give a hint, however, cannot be used solely for diagnosis [38]. Prostate secretions can be obtained by digital-rectal massaging of the gland, while a urinary catheter is placed at the site of the pars disseminata. The secretions can be aspirated, provided the urinary bladder was emptied and flushed before; they can be examined cytologically and bacteriologically [19]. Collection of more prostate gland epithelial cells is possible by use of the urethra-brush method. The brush is introduced in the urethra while hidden in a plastic catheter; at the site of the prostate gland, the brush is pushed forward several times and then sterile retracted inside the catheter and outside the urethra. The brush and the collected fluid are deposited in sterile sodium chloride solution and centrifuged, the pellet can be examined cytologically and bacteriologically [40].

In dogs, the final diagnosis BPH can only be made by use of fine needle aspiration (FNA); this method should be performed when the dog is sedated and received analgesia. The FNA is done transcutaneously under sonographical control [8, 19]. However, even though providing the final diagnosis, this method is mostly not necessary. The cytologically obtained results correlate well with histopathology. Only for differentiation between chronical prostatitis and prostate gland carcinoma, FNA or biopsy must be performed [66, 72–74].

Measurement of the canine prostate-specificesterase (CPSE) can be helpful. The concentration of this enzyme in the blood is significantly increased in case of canine BPH and other diseases [29, 33, 48, 75–78]. Unfortunately, it is not possible to differentiate between BPH, prostatitis, and neoplasia, and the reference values for healthy dogs are variable in the literature [48, 75, 77]. The secretion of the CPSE is age-dependent in dogs, therefore, reference values must be critically considered. The diagnosis BPH should not be solely based on measurement of the CPSE. In one study, a combination of clinical symptoms, CPSE measurement, and calculation of the prostate volume (real volume/estimated volume = V ratio) were evaluated. The clinical BPH coincided with a V-ratio of >2.5 and a CPSE concentration of > 90 ng/ml; the sensitivity was 85% and the specificity 72% [48]. Meanwhile, a commercial assay is available (Odelis® CPSE, Bio Veto Test, Nice, France) and another study revealed a sensitivity of 97.1% and a specificity of 92.1% [79].

4.1.3 Differential diagnoses

When the general condition is undisturbed, BPH can be mistaken for chronical prostatitis or beginning neoplasia in dogs.

4.1.4 Therapy

Therapy is only necessary when clinical symptoms are visible. When the dog is asymptomatic, regular clinical and sonographical controls every 3–6 months are recommendable [42]. Vets can choose between different medicaments, providing the best choice for a subject [7].

The most effective method is the castration, involution starts within 6–12 weeks [7]. The clinical symptoms will disappear earlier and a decrease in size can mostly be palpated after 1–2 weeks [42]; the volume will decrease to 60% within one week [10], and by 50% after three weeks [39]. Bloody preputial discharge disappeared in 89% of cases within 4 weeks after castration [38]. Castration is the treatment of choice in case of hyperdistention, dyschezia, perineal hernia, or large retention cysts [42].

Table 1 provides an overview of useful and recommendable medicaments against BPH

Medicaments with an antiandrogenic effect likewise and rapidly reduces the size of the gland. They competitively block the binding of testosterone to its receptors and decrease libido within 3 days. One example is cyproterone acetate, furthermore delmadinone acetate. Some preparations are not licensed for use in animals. These medicaments caused a reduction in canine prostate gland size by 28% within two weeks [82], and the clinical symptoms improved earlier. The duration of effectiveness is approximately 6 months when an average dose of 3 mg/kg is chosen. Side effects in male dogs are a latent diabetes mellitus and diseases of the mammary gland (tumors, hyperplasia, cysts, and galactorrhea).

For breeding dogs, medicaments not decreasing the libido are desirable, enabling examination of the semen quality while the dogs are still under treatment. for example osaterone acetate is a gestagene with anti-androgenic effect. It decreases the uptake of DHT in the prostate gland and decreases the activity of the 5α-reductase. Osaterone acetate furthermore suppresses the nuclear DHT- and androgen-receptor expression in the gland [86]. The size of the gland was significantly reduced to 62.6% within 7 days when a daily oral dose of 0.2–0.5 mg/kg was given [10]. A daily oral dose of 0.25 mg/kg for 7 days reduced the size to 64.3% within 14 days [84]; the testosterone concentration was significantly reduced for 3 months [10], then slowly increased, which is believed to point toward a low-grade anti-gonadotrophic effect [87]. The semen quality was low grade decreased during the therapy; the volume was decreased for 4 months. An increase in the percentage of morphological changings was observed 4 weeks after beginning of the therapy and during the following 1.5 months [10]. This medicament is recommendable for breeding dogs because of its rapid effect and the maintained libido. Within three months after beginning of the therapy, the sonographical appearance of the gland and the quality of the ejaculate are back to normal. Some side effects were observed: an increase in appetite for 1–3 weeks (3/15), lethargy (2/15), and low-grade loss of hair (1/15) [7].

This medicament is applicated orally; in case of vomiting, it is therefore not recommendable. In this case, injectable preparations are available for dogs.

In case of mild BPH, the 5α-Reductase-Inhibitor Finasteride is effective (for example Proscar® 5 mg Tabl. Merck, Vienna, A) [12]. Doses for dogs and duration of application are variable in the literature (Tab. 2) [42, 83]; however, the tablets should be given for 3–4 months. Since semen quality and libido are not changed by the medication, it is recommendable for breeding dogs [12, 83]. Finasteride is a teratogenic substance; nevertheless, fertility and resulting puppies are not concerned [85]. Side effects are not described.

Agent	Effect	Preparations	Dosage	Application	Decrease in size after (days)	Duration of efficacy (months)	Side effects	Authors
Cyproterone-acetate	AntiAndrogen	Injectable (Depot)	2–5 mg/kg SID (can be repeated after 1 week)	s. c.	7–14	6	Apathy, thirst, mammary tumors, increase in appetite, loss of libido	[31, 80, 81]
		Tablets	2–3 mg/kg daily	p.o.				
Delmadinone-acetate	AntiAndrogen	Injectable	1–3 mg/kg SID (can be repeated after 1 week)	i. m.	14	6	Diabetes mellitus, mammary tumors, increase in appetite, loss of libido	[82, 83]
Osaterone-acetate	AntiAndrogen	Tablets	0.2–0.5 mg/kg/day (7 days)	p. o.	7–14	6	Decrease in semen quality, increase in appetite, loss of hair, lethargy	[7, 10, 84]
Finasteride	5α-Reductase-Inhibilor	Tablets	0.1–0.5 mg/kg/day (16 weeks)	p. o.	30–120	Dependant on duration of application	-	[12]
			1 mg/dog/day (3–21 weeks)	p. o.				[85]
			1 mg/kg/day (3 weeks)	p. o.				[42, 83]
			1.25 mg /dog /day (195 days)	p.o.				[42]
Deslorelin	GnRH-Agonist	Subcutaneous implant	4.7 or 9.4 mg /implant (repeated application possible)	s. c.	37	6–12	Flare-up within 1 week	[51]

Table 1.
Overview of useful and recommendable medicaments against BPH.

For prolongation of an anti-androgenic therapy, long-lasting agonists of the gonadotropin-releasing hormone (GnRH) are suitable for dogs with BPH. Subcutaneous implants containing, for example, deslorelin (Suprelorin® 4.7 or 9.4 mg, Virbac, F) are licensed for male dogs and male ferrets. Many studies using different GnRH agonists and dosages are available, but difficult to compare [7]; however, deslorelin is the only licensed preparation. After resorption of a certain amount of GnRH, down-regulation of the GnRH receptors in the pituitary gland leads to a decrease in the secretion of the gonadotropins "follicle stimulating hormone" (FSH) and "luteinizing hormone" (LH), and consecutively to a decrease in the secretion of testicular testosterone by 90% and the spermatogenesis. The volume of the prostate gland decreases within 6 weeks by 50%, when a 4.7 mg implant is used [7, 51, 88], beginning after 37 days [51].

In dogs, the initial therapy leads to an increase in testosterone secretion; this flare-up can be suppressed by oral application of an antiandrogen. This is important in case of an acute enlargement of the gland with acute symptoms [7].

GnRH antagonists can be used for therapy of canine BPH, unfortunately, the second generation of these drugs caused anaphylaxis in some cases. Meanwhile better agonists, which are potent, long-acting, and without side-effect, are available; however, they are only licensed for use in humans. Acyline is a preparation of the third generation and was used in one study at a dose of 330 mg/kg s.c. in dogs, leading to a reversible decrease in FSH, LH, and testosterone over 9 days. When a long-acting GnRH-agonist was used in dogs, Acyline successfully prevented a flare-up. In addition, the prostate volume was decreased by 38% after 30 days, echogenicity and heterogeneity were decreased, and the resistency-index (Doppler sonography) was normal again [89]. Monthly injections are required, rendering this medicament for short-term and exceptional use only. Further investigations with long-acting preparations would be of interest. Other medicaments like estrogens, antiestrogens, aldosterone-receptor antagonists, alpha1A-adrenerge-receptor antagonists, phosphodiesterase (PDE)-5 inhibitor, vitamin D receptor agonists, and intraprostatic injection of botulinus toxin type A (BT-A) were investigated; however, they are now obsolete or proved to be ineffective [7].

4.1.5 Prognosis

In dogs, the clinical symptoms can be effectively treated; however, the course of the disease is recurrent. Castration will finally resolve the problem. In stud dogs, special medicaments not decreasing the libido are available and fertility prognosis is good.

4.1.6 Prophylaxis

Regular clinical and sonographical controls of the dogs are a good prophylaxis since only treatment or castration in time will prevent the disease. These controls are recommendable when the dog reached 40% of its estimated lifetime [18].

4.2 Prostate gland cysts

4.2.1 Causes

Cystic changes of the canine prostate gland (intraprostatic cysts) mostly develop in the aged gland, changed by BPH, because of accumulation of prostatic secretions in the dilated prostatic acini; furthermore because of obstruction, compression of intraprostatic channels, or accumulation of urine, when a connection between the cyst and the

urethra exists [90, 91]. Paraprostatic cysts are dilated residua of the Wolff channels; they can be situated in the cranio-lateral, ventral or caudal region of the prostate, and reach a remarkable size. In some cases, they become mineralized [90, 92]. Secondary infections and abscesses can be complications. In one study the prevalence of prostatic cysts was 14% (12/85) and 42% out of these were secondary infected [90].

4.2.2 Clinical findings

The symptoms are dependent on the disease. Many small intraprostatic cysts are asymptomatic in dogs until the enlarged gland causes problems. Intraprostatic cysts frequently occur in the course of BPH and prostatitis; later on, they can cause enlarged abdomen, abdominal pain, decreased well-being, and in case of rupture or secondary infection, an acute abdomen, and sepsis.

4.2.3 Diagnosis

In dogs, diagnosis should be done by sonography or X-ray. Sonographically, cysts appear as hypo- or anechoic, round structures with a thin wall, sometimes sediment or internal cysts can be visualized [45] (**Figures 4** and **5**). The cysts can be punctured and the contents examined cytologically and bacteriologically.

4.2.4 Differential diagnoses

As described in the chapter BPH.

4.2.5 Therapy

Canine intraprostatic cysts up to 3 cm in diameter can be treated with a 5α-reductase-inhibitor (Finasteride) or with anti-androgens; mostly they regress

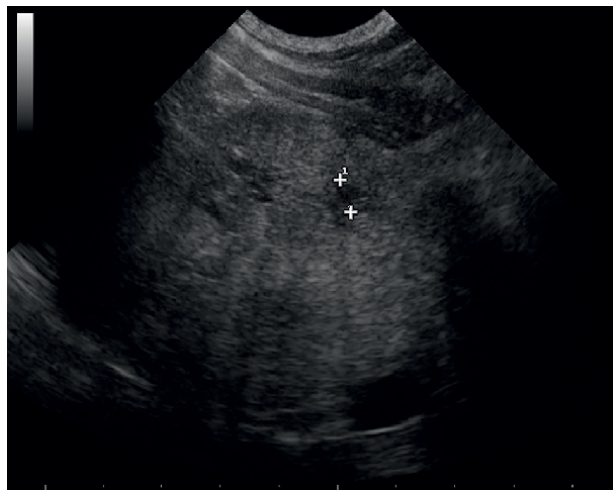

Figure 4.
Sonography (B-mode) of a prostate gland with BPH and intraprostatic cysts. A small cyst is visible (white crosses). Cysts up to 3 cm in diameter can regress with anti-androgen therapy. Cysts filled with urine have a higher recidive rate, also after puncture.

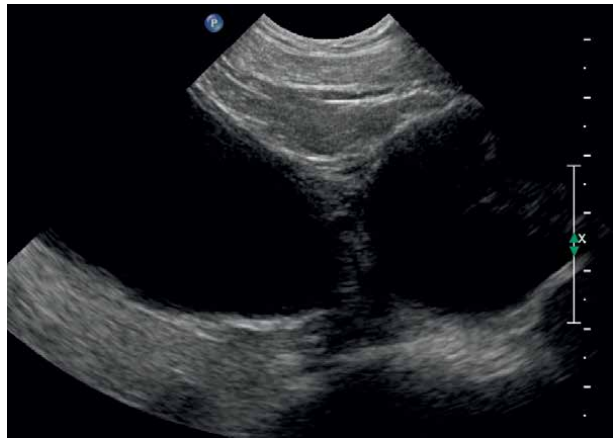

Figure 5.
Sonography (B-mode) of a paraprostatic cyst. On the left side, the urinary bladder is visible; the mucus membrane is irregular and thickened. To the right, a paraprostatic cyst is situated, filled with hypoechoic fluids. The dog showed mild symptoms of a BPH with bloody preputial discharge and stranguria.

within 2–3 weeks. When the treatment is ineffective or in case of larger cysts, they have to be punctured and the secretions aspirated or the cysts must be surgically removed. The puncture should be done with the aid of sonography and transcutaneously (**Figure 6**). The treatment mostly has to be repeated one to four times and only if these measures stay without success, the operation should be considered [93]. The ultrasound-guided percutaneous drainage with alcohol sclerotherapy is controversial,

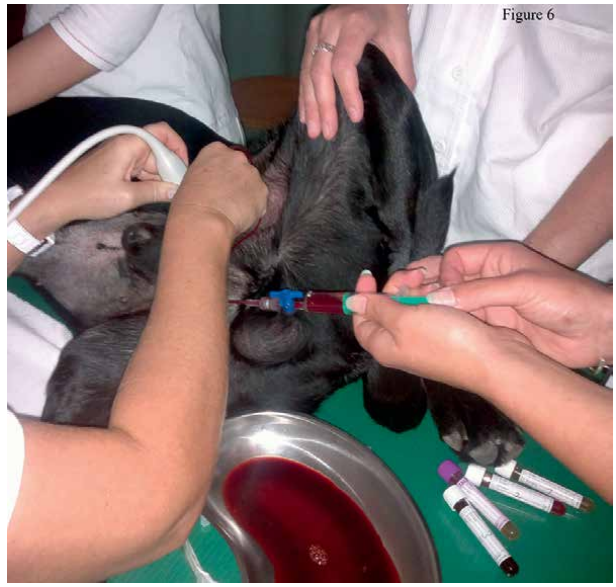

Figure 6.
Transabdominal puncture of a prostate abscess. The gland was visualized with a 7.5 MHz convex probe, the cyst was punctured with a 0.9×40 mm needle, connected to an extension and a three-way cock. The contents were sucked off with a sterile syringe and examined cytologically and bacteriologically.

even though some reports are promising [94]. Recently, canine autologous platelet-rich plasma (PRP) obtained through separation of liquid and solid components from whole blood, it was instilled after removal of cystic fluid in dogs with BPH and prostatic cysts [95]. The PRP dose was half the fluid removed from the cyst. Sixty days later, the cysts were no longer detectable sonographically. The PRP is known to affect antibacterial, analgesic, and anti-inflammatory [96]. The surgical treatment and the treatment of abscesses will be discussed in the following chapter.

4.2.6 Prophylaxis

As described in the chapter BPH

4.3 Inflammatory diseases of the prostate gland

4.3.1 Causes

In dogs, inflammatory diseases can be acute or chronical; they are mostly complicated by infections that ascend via the urethra or spread via the blood circulation [39, 97]. Prostatitis therefore may occur in both castrated and intact dogs. In some cases, a BPH, squamous metaplasia or neoplasia is complicated by an infection. In one study, in 66.6% of male dogs with clinical BPH, bacteria were isolated in the sperm-rich phase of the ejaculate; out of these, 61.1% were positive for mycoplasms, and out of these, 54.5% were positive for *Mycoplasma (M.) canis* [36]. In 2/3 of all cases of prostatitis, a mixed bacterial culture can be found, and only in 1/3 of patients a monoculture [98]. Infectious agents mostly are *E. Coli*, Staphylococcus spp., Streptococcus spp., Proteus spp., Pseudomonas spp., Klebsiella spp., *Brucella canis,* etc., as well as anaerobe germs; Seldom are viruses like the canine distemper virus, or blastomyces and cryptococci in urine, semen, or prostate secretions [9, 39, 97, 99]. It is important to know that abscesses in the prostate gland can be infectious or sterile [7, 41, 99].

4.3.2 Clinical findings

The acute prostatitis can cause severe symptoms like acute anuria or obstipation. A frequent symptom in dogs is purulent-bloody preputial discharge. Fever, inappetence, vomiting, and diarrhea are possible. In case of an abscess, palpation of the gland is highly painful and fluctuation is typical; rupture will cause septic shock.

The chronical prostatitis usually starts with symptoms of the BPH, and then the course is recurrent, causing loss of weight and shaggy hair. Superinfections frequently occur.

4.3.3 Diagnosis

The diagnosis should be done by clinical examination of the dog, sonography, and examination of urine and semen inclusive bacteriological examination. In addition, prostate secretions and the contents of cysts can be examined cytologically [19]. Rectal palpation will be painful. The gland can be asymmetric; the consistency will be elastic in case of acute inflammation, in case of chronical inflammation increased and sometimes hard, the surface can be uneven.

In dogs, hematuria and bloody preputial discharge frequently occur, and pyuria or purulent discharge may occur in case of prostate gland abscess. Bacteriological examination is mostly positive [41].

Blood picture: in case of acute prostatitis and abscesses, leucocytosis and neutrophilia are frequent, in chronical prostatitis, these findings may be lacking. An increased concentration of the enzyme canine-prostate-specific esterase (CPSE) may indicate a prostatic disease; however, differentiation between BPH, prostatitis, and neoplasia is not possible in dogs. Furthermore, the literature provides variable cut-off values [48, 75, 77] and the secretion of the CPSE is age-dependent in dogs. The measured values, therefore, have to be carefully interpreted; the diagnosis must include other findings.

Semen collection in case of acute prostatitis will not be possible but may be helpful in case of the chronical prostatitis. The semen quality initially shows the same abnormalities as in BPH and will decrease in case of infection. Admixture of erythrocytes is a frequent finding, furthermore decreased motility and an increase in morphological abnormalities [19, 39, 40]. The bacteriological examination of the semen or prostatic secretions is mostly positive [7, 9, 39]; additional cytological examination of the prostatic secretions is useful, in case of acute prostatitis and abscesses, granulocytes, blood cells, and bacteria are frequently found, whereas prostate cells appear normal [42, 99].

In dogs, the cytological findings correlate well with the patho-histological findings [39]; however, not with the bacteriological findings [100]. Collection of prostatic secretions is not sterile because of the physiological mixed flora in the urethra [101]; therefore, the quantitative bacteriological findings have to be considered as well.

The transcutaneous, sonographically guided fine-needle-aspiration (FNA) of the prostate tissue and puncture of fluid-filled cysts are important for differentiation between canine BPH and chronical prostatitis or neoplasia [39, 41, 44, 102] (**Figure 6**). The collected material should be examined cytologically and bacteriologically. Up to 70% of prostatitis cases were correctly diagnosed by use of FNA [102]. Complications rarely occur; in some cases, low-grade bleeding and inflammation were observed, especially in case of inflammatory changings [93]. Even though at the time of puncture or FNA it is not known, whether the obtained material is infectious or not, the procedure is safe for the patient, when performed in a sterile manner. The dog should receive nonsteroidal anti-inflammatory drugs (NSAID) for 3 days after the puncture and should be treated as soon as possible with suitable antibiotics according to the resistance test. In rare cases, spreading of tumor cells is possible [103].

B-mode-Sonography: in dogs, enlargement, asymmetry, and heterogeneity are prevailing symptoms. In case of acute prostatitis and abscesses, hypoechogenic sites can be found (**Figure 7**); in chronical prostatitis, hyperechoic sites are frequent, and in case of neoplasia also mineralization (**Figure 8**) [45, 63, 104].

Unfortunately, it is not possible to differentiate between chronically inflammatory and tumorous changings, not with B-mode and Doppler sonography; in these cases, an FNA or biopsy is obligatory in dogs [8, 44, 73]. With grey-scale or pulse-wave Doppler-sonography, it was not even possible to differentiate between inflammatory and normal canine tissue [105]. Similarly, other imaging methods like CT or MRI cannot provide a secure diagnosis; however, in case of canine prostatitis, the CT findings correlated well with the CT outcome [68]. When using CT, the age of the dog must be considered since the normal CT findings change in the aging dog. The prostate growth shows three phases [106]: during the first phase (1–5 years), the gland reaches normal morphology; in the second phase (6–10 years), first hyperplastic changings occur; and in the third phase (≥11 years), senile involution is typical. These changings can be observed in the CT pictures as well [67].

As described in the chapter BPH, the echostructure analysis revealed typical findings in case of prostatitis; homogeneity was significantly decreased in comparison to BPH [62]. Further investigations are necessary to prove these first results.

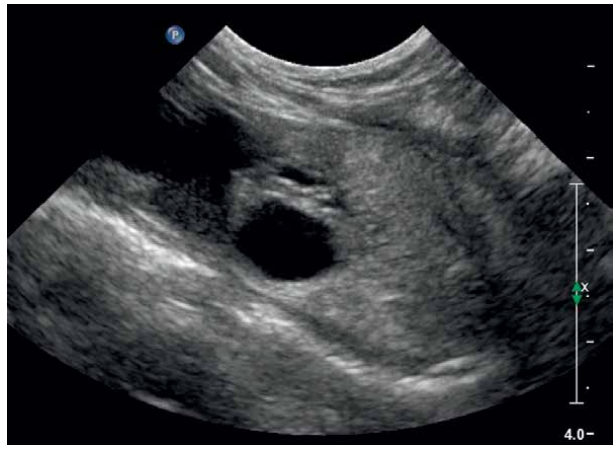

Figure 7.
Sonography of a prostate gland with acute prostatitis. Prostate gland of a 12-year-old dog with fever, apathia, urine loss, obstipation, and a painful abdomen. The prostate gland was painful upon digital-rectal palpation. The gland was high-grade enlarged and the structure was inhomogenous. An intraprostatic cyst, 1,5x2 cm in size, was visible. The urine was examined bacteriologically and Streptococcus canis +++ was found. The dog received effective antibiotics according to the resistency test for 6 weeks and the antiandrogen cyproterone acetate (3 mg/kg SID, s.c.). A sonographical control 2 weeks later showed that the cyst had diminished.

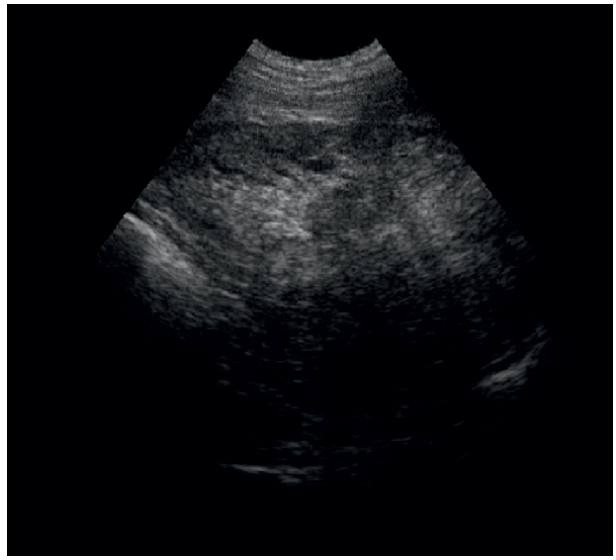

Figure 8.
Sonography of a chronical prostatitis. The gland was high-grade enlarged and the structure was inhomogenous, mainly most areas hyperechoic. Very small cysts were visible. The dog showed chronic recurrent bloody preputial discharge, dyschezia, and obstipation. The semen was examined bacteriologically and ++ E. coli was isolated.

4.3.4 Differential diagnoses

BPH and neoplasia of the prostate gland have to be considered.

4.3.5 Therapy

In acute canine prostatitis, typically, high-grade disturbance of the general conditions occurs; furthermore, acute urination and defecation problems require emergency measures. The rapid reduction of the prostate gland size is important, in addition to effective treatment of the infection. Intravenous infusions of physiological solutions are necessary for treatment of circulatory disturbances. Drugs against pain and inflammation such as NSAID and/or morphine derivates should be given (for example Carprofen 4 mg/kg SID i.v. or Buprenorphin 0.01–0.02 mg/kg every 6–8 h i. v.). In case of vomiting, metoclopramide injections are useful (0.5–1 mg/kg, BID-TID, s. c., i. m., i. v.) or maropitant (1 mg/kg SID s. c., i. v.). Dogs should be In-patient while treated until improvement.

Antibiotics have to be chosen according to a resistance test and according to the ability to penetrate the diseased tissue. In acute cases, the blood-prostate barrier is ruptured; therefore, each broad-spectrum antibiotic can be applied when effective according to the resistance test [97, 107]. Meanwhile, it is important to not only examine for bacteria but also for mycoplasms (M.) and ureaplasma (U.) inclusive specification and quantification; *M. canis*, *M. cynos,* and *U. canigenitalium* were isolated in semen, prostate secretions and urine of dogs with prostatitis. Even though it is not proven that they are causative agents, high-grade monocultures should be treated according to a resistance test [97, 99, 108]. Acute symptoms may be treated with broad-spectrum antibiotics before the resistance test is available [7]. In these cases, fluoroquinolones (for example Enrofloxacin s. c, i. v. SID or SOD 5–10 mg/kg) or erythromycins (for example Azithromycin 5–10 mg/kg SOD) can be given. These antibiotics are also effective against mycoplasms and ureaplasma.

In chronical canine prostatitis, the blood-prostate barrier is intact; therefore, antibiotics must be chosen according to the resistance test and the ability to penetrate the tissue. The latter is possible by using weak alkaline medicaments with a high pKa-value (acid-dissociations constant), good fat solubility, and weak protein binding [97]. In these cases, fluoroquinolones and erythromycins are good options as well, furthermore clindamycin and chloramphenicol.

In both acute and chronical prostatitis, the duration of treatment is important; in chronical cases, 4–6 weeks and up to 8–12 weeks are recommendable in dogs [19, 107]. One week after the end of the antibiotic treatment, another bacteriological examination should be done [19].

In dogs, prostate abscesses can be punctured and emptied; for this measure, a mild sedation is required. The needle should be carefully placed under sonographical control and samples for cytological and bacteriological examination obtained (**Figure 6**); sometimes one to four repetitions are required and in some cases, operative removal of the abscess is necessary [93]. Operative treatment is possible by marsupialization, a Penrose drain, or partial prostatectomy [109–112]; a further method with low recidivism rate is the operative drainage of the abscess cavity and consecutive filling with omentum (omentalization) [112]. The prostate has to be pulled out of the abdomen; the contents of the abscess are sucked off (**Figure 9a** and **b**), then the opening is enlarged and the cavity flushed. Another opening is cut into the opposite side of the gland (**Figure 9c**) and the omentum is pulled into and through the cavity. The omentum is fixed with a suture on the opposite side of the gland. Additional application of antiandrogens and antibiotics according to a resistance test are necessary measures.

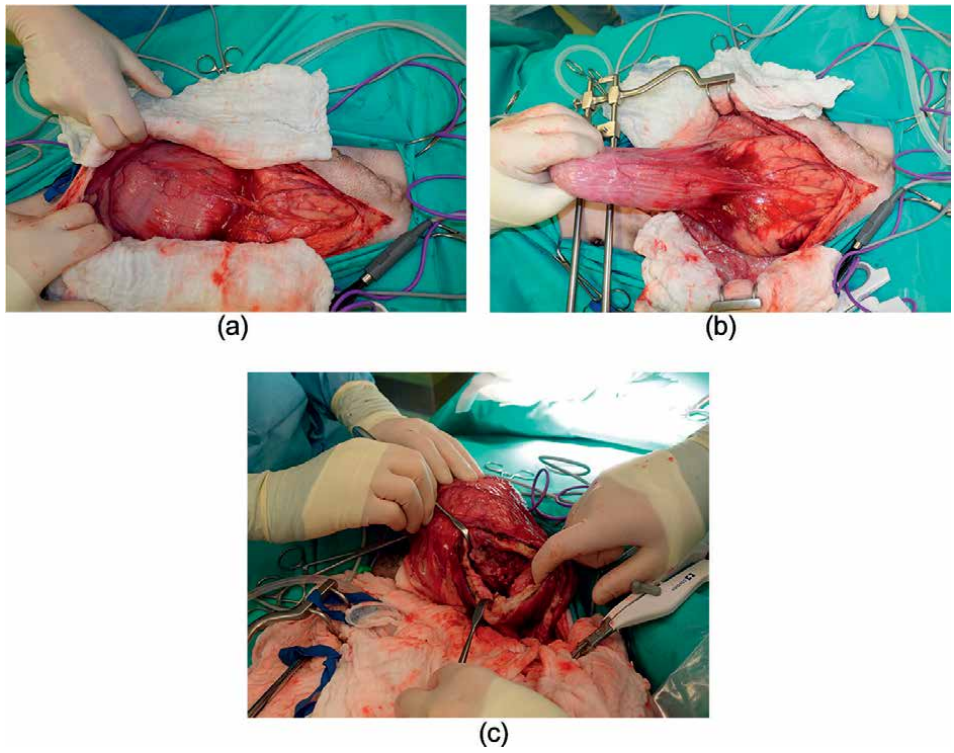

Figure 9.
Omentalisation of a paraprostatic cyst (a); The huge paraprostatic cyst was situated behind the urinary bladder (to the right, black arrow). The wall was high grade thickened. (b) The urinary bladder was emptied and the prostate cyst was pulled out of the abdomen (c) After puncture of the cyst the contents were sucked off, then a large piece of the wall was removed on both sides of the cyst by using a sealing device (LigaSure™, Medtronic, Vienna, A). The omentum was pulled into the cavity and fixed on both sides of the cyst by using resorbable material.

4.3.6 Prognosis

The clinical symptoms can be effectively treated in both acute and chronical cases; however, the course of the disease is recurrent. Fertility prognosis is good when treated in time, with well-chosen medicaments, and over a sufficient period.

4.3.7 Prophylaxis

Regular clinical and sonographical examinations, starting when the dog reached 40% of the estimated life expectation, are recommendable [18].

4.4 Squamous metaplasia

Squamous metaplasia develops because of an endocrine active testicular tumor, secreting androgens and causing hyperestrogenemia, but in addition because of exogenous estrogens [113]. The metaplasia causes a morphological change in the gland, sonographically resembling an inflammation; sometimes cysts occur.

The disease is a side effect of hyperestrogenemia since this problem causes the clinically relevant changes in the blood picture and causes organ damages. Typical

symptoms are alopecia, hyperpigmentation of the abdomen and inguinal region, gynecomastia, and in severe cases anemia.

The diagnosis can be made with FNA; however, rapid diagnosis and treatment of the hyperestrogenemia are more important. Mostly castration will solve the problem. However, it is important to know that the hyperestrogenemia can persist for months after the removal of the testicular tumor. Recurrence of the problem after castration may point toward metastasis.

4.4.1 Prognosis

When the dog is castrated, the estrogen concentration slowly decreases over weeks and months. During this time, infections frequently occur. Dependent on the degree of anemia and organ damage, a careful prognosis is appropriate. In case of low-grade changings and correct treatment, the prognosis is good.

4.5 Tumors of the prostate gland

4.5.1 Causes

Prostate gland tumors are seldom in dogs (<1%), [16, 114] and mostly malign adenocarcinomas or transitional cell carcinomas and seldom lymphomas [41]. The cells of origin sometimes are not identified [15, 115]. They are more frequently diagnosed in castrated than in intact dogs, the growth is not androgen-dependent [14, 15, 116]. Other diseases of the prostate gland are not predisposing [19]. It is not known, whether the age at castration plays a role [16]. However, the age itself is an important factor, since the disease mainly is diagnosed in dogs aged > 8 years [51, 117–119]. Medium to large size breeds are more frequently concerned than smaller or toy breeds [14, 114]. A breed disposition is not proven; however, a higher risk/odds ratio was found for Shetland Sheepdog, Scottish Terrier, Bouvier des Flandres, Doberman, and mongrels [9, 14, 120].

Recent studies investigated changes in the prostate gland during cancer development at the molecular level. A lack of androgen receptor and the overexpression of P-glycoprotein (P-gp) was described, indicating that androgens do not play an important role in pathogenesis [116]. P-glycoprotein regulates the influx and efflux of testosterone in prostatic cells. New findings suggest NF-kb dysregulation as a probable factor contributing to oncogenesis; chronic inflammations may trigger the change in precancerous cells causing DNA and epigenetic damage [121]. NF-kb is an inducible cytoplasmic transcription factor, able to activate genes for inflammatory cytokines, adhesion molecules, enzymes related to inflammation (such as cyclooxygenase-2), telomerase, antiapoptotic proteins, and cell cycle-regulatory genes [121].

The growth is most aggressive with an invasion of surrounding tissues and high metastatic potential. The incidence of metastases varies between 16% and 80%, dependent on age [119, 122]; the sites of metastasis are primary the lung, then regional lymph nodes, liver, urethra, spleen, colon and rectum, urinary bladder, bones, heart, kidney, and adrenal gland, but also the skin [123]. Metastases are mostly already present at the time of diagnosis of the prostate gland tumor [19].

4.5.2 Clinical symptoms

The symptoms vary independently on castration status; in some dogs, gastrointestinal symptoms are predominant (defecation problems, tenesmus), in others,

symptoms of the urogenital tract occur first (stranguria, hematuria, incontinence, dysuria, pollakisuria, and polydipsia). Enlargement of the gland was observed in only 45% of cases [119]. In some dogs, lameness, loss of weight, and abdominal pain become obvious, especially, when metastases occur [9, 114].

Since the symptoms are unspecific, a prostate gland tumor must be considered in aged dogs with severe symptoms of a disease of the urogenital tract and gastrointestinal symptoms [16].

4.5.3 Diagnosis

Prostate gland tumors are frequently diagnosed too late when the aggressive invasive growth already caused massive tissue damage and metastases. Accompanying inflammation and secondary infections of the urogenital tract complicate the diagnosis. However, early detection is an important factor for survival.

Digital rectal palpation may reveal an uneven surface, immobility, asymmetry, and/or painfulness. A blood picture can show neutrophilia, leucocytosis and in 70% an increase in alkaline phosphatase concentration. Pyuria and hematuria are possible; in the sediment, tumor cells can be detected [119].

However, cytological examination of urine or prostate secretion sediment is unreliable, even when the cytobrush method was used. For the final diagnosis, a biopsy and histological examination of the tissue are obligatory. Transcutaneous FNA has a sensitivity of 80% [119], which can be increased to 89% by punch-biopsy or excisional biopsy [102, 103, 124]. For punch-biopsy or excisional biopsy, total anesthesia is required. The gland has to be pulled forward to be able to perform the biopsy on the ventrolateral surface. The wound is closed with single sutures, including the capsule and parenchyma [125]. Histologically, a prostatic adenocarcinoma can be differentiated from a prostatic carcinoma, urothelial, and tumors of mixed morphology [124, 126]. A possible side effect is the spread of tumor cells [103].

Sonography is not useful to differentiate between inflammation and neoplasia; however, can be helpful [45, 63]. With B-mode, the gland appears inhomogeneous, with hyperechoic areas; mineralizations are frequent and the borders in > 80% of cases appear irregular and diffuse against the rectum, and sometimes even rupture. In many cases, the regional lymph nodes are changed [119] (**Figure 10**).

Some imaging methods were improved. With contrast-enhanced-Doppler sonography it is possible to visualize the perfusion in the normal prostate tissue and to compare it with prostate neoplasia; in case of adenocarcinoma, the perfusion was significantly higher [81]. Elastography was used in one Labrador dog with prostatic adenocarcinoma, and the histological result of the FNA correlated well with the findings of the elastography [121]. A new experimental method is a combination of simultaneous magnet-resonance spectroscopy (MRS), positron-emission-tomography (PET), and multiparametric magnet-resonance (mpMR). In one study, the results were compared with findings from transrectal sonography and prostate biopsy. In 3/3 dogs, tumor growth was diagnosed by using the combined method; the diagnosis was verified by biopsy [71].

An X-ray of thorax and abdomen should be done to diagnose metastases in the lymph nodes, pelvic bones, and the lung [11, 41].

Recent studies focus on the detection and development of biomarkers for canine prostate cancer [126, 127]. Markers are not easy to find in case of canine prostate cancer since the tumor growth is aggressive and the pattern variable, the basal cell layer is discontinuous and markers are frequently absent. A combination of markers might

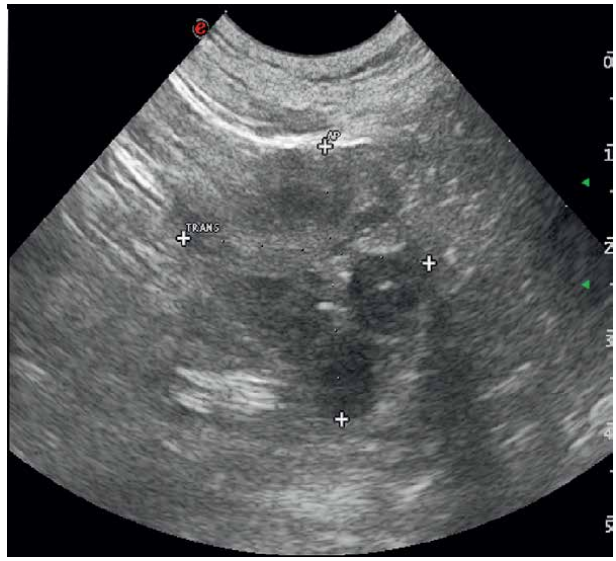

Figure 10.
Sonography of a carcinoma of the prostate gland (B-mode). The male dog showed chronical prostatitis, loss of weight, and a matt coat. The prostate gland was only low-grade enlarged, but high-grade inhomogeneous, with mineralizations. The margin was not well defined and could not be separated optically from the wall of the rectum. Small intraprostatic cysts were visible. FNA of the gland was performed and revealed the diagnosis of prostate carcinoma.

increase the diagnostic accuracy [127]. For a precise immunohistochemical analysis, different markers are necessary to differentiate between urethral, glandular, or ductal origin of the tumor, which is possible in human medicine but not sufficiently investigated in the dog. In dogs, the prostate cancer most probably originates mainly from collecting ducts [128]. In one study, qPCR revealed increased expression of PSMA in all cancer tissues [128].

In dogs, both urothelial carcinoma of the lower urinary tract and prostate cancer may occur. In both cancer types, *canine (c) BRAF V595E* gene mutations were found. The *BRAF* genes belong to the *RAF* gene family known to contribute to the MAPK pathway; mutations promote growth of cancer cells during oncogenesis [129]. *Canine(c)BRAF V595E* gene mutations were recently detected by means of droplet digital PCR (ddPCR) in approximately 80% of urogenital cancer in dogs. The *cBRAF* mutation was detectable in urine samples with the same sensitive assay and in 75% of the cancer patients [129, 130]. However, since in approximately 20% of canine urogenital cancer the *cBRAF* mutation is not detectable, the sensitivity of the ddPCR assay does not exceed 80%.

To differentiate between urothelial and prostate carcinoma, a combination of markers will be necessary. In a recent study [131], the chemokine CCL17 was found to contribute to regulatory T cell (Treg) recruitment in prostate tumors. In dogs with prostate cancer, tumor-infiltrating Tregs were found to be associated with bad prognosis [132]. In urine samples of dogs with urothelial cancer, increased concentrations of CCL17 were found in comparison to healthy dogs. The *cBRAF* mutation is believed to induce the COX-2/PGE_2/EP2 pathway, thereby triggering the CCL17 production and the Treg infiltration in canine urothelial carcinomas; however, a direct relation between *cBRAF* mutation and prognosis was not possible [131]. Recently, the concentration of another chemokine, named CCL2, was found to be increased in urine

of dogs with urothelial carcinoma [133]. The combined measurement of CCL17 and CCL2 in urine might improve the sensitivity and specificity of each biomarker for detection of canine urothelial cancer [131].

In one study, RNA-Sequencing of canine normal prostate gland tissue and malignant tissues was performed to find differentially expressed genes (DEGs) and deregulated pathways. The detected DEGs were grouped into the superior pathways (1) inflammatory response and cytokines; (2) regulation of the immune system and cell death; (3) cell surface and PI3K signaling; (4) cell cycle; and (5) phagosome and autophagy. Meanwhile, some genes were listed in relevant databases and might improve diagnosis and therapy in future.

Furthermore, canine prostate cancer cell lines have been developed making investigation of molecular mechanisms easier [134]; one cell line expressing red-fluorescence proteins was developed to improve in-vivo imaging [135].

4.5.4 Differential diagnosis

BPH, chronical prostatitis, or other tumor diseases must be considered, especially in case of weight loss.

4.5.5 Therapy

Conservative therapy comprises chemotherapy and palliative measures and shall improve the median survival time (MST) and well-being. Surgical treatment is possible; partial and total prostatectomy followed by chemo- and radiotherapy, photodynamic therapy and COX inhibitors [15] are possible methods. Castration is not useful and should not be recommended [19, 119].

Prostate surgery is mostly recommended in case of intracapsular growth and early-stage cancer. For total prostatectomy, the prostate-inclusive prostatic urethra has to be removed; thereafter, the urethra is reconstructed. Subtotal intracapsular prostatectomy proved to prolongue the MST more than 5fold in comparison to total prostatectomy (112 ± 63.3 days vs 19.9 ± 10.67 days) [136]. Most frequent postoperative complication is a permanent incontinence, occurring in 33–100% of cases; however, less frequent after subtotal intracapsular prostatectomy [136–138]. In one retrospective study [139], the postoperative survival time (time between operation and death) was 231 days (median; range: 24–1255 days). In the evaluated studies, ureter-urethral anastomoses (14), cysto-urethral anastomoses (9), anastomoses between ureter and colon (1), and anastomoses between urinary bladder neck and pelvic part of the urethra were described (1). The dogs in addition received mitoxantrone, NSAID, metronomic thalidomide, cyclophosphamide, piroxicam, carboplatin, and/or deracoxib. In 8/23 dogs, postoperative incontinence occurred. Further complications were dehiscence of sutures, uroabdomen, and prepubic herniation. In 3/23 dogs a recidive occurred, in 4/23 metastases were diagnosed [139].

Another study compared the outcome of medical therapy (n=12) and surgery in dogs with adenocarcinoma of the prostate gland [140]. The surgery comprised total prostatectomy (TP, n=20) and prostatocystectomy (TPC, n=9). In the surgical group, the overall MST was longer than in the medical treatment group (337 vs. 90.5 days). Within the surgical group, the postoperative MST was longer in the TP group (510 vs. 83 days). In case of aggressive prostate cancer, TPC is preferred, therefore more severe complications occur, explaining the shorter MST.

In recent years, the surgery was improved by use of Light-Amplification by Stimulated-Emission-of-Radiation (laser). Meanwhile, the method is used for prostatectomy. The laser (Diode, Nd:YAG or CO2) must be adapted to the predominating tissue, i.e. the vascularization and the pigment since the absorption spectrum can be influenced by melanin, hemoglobin, and water. For prostatectomy, the CO_2 laser in combination with electrocautery was proven advantageous [141].

Immunotherapy is under intense investigation in human medicine and recently, a promising study in dogs with naturally occurring prostate cancer was published [132]. In this study, the presence and molecular mechanism of targeting regulatory T-cells (Tregs) were studied in canine cancer cells and an anti-Treg treatment (anti-human CCR4, mogamulizumab) in combination with Piroxicam tested in dogs with prostate cancer. The tumor response was evaluated according to canine response evaluation criteria [142]. The presence of tumor-infiltrating CCR4 Tregs was found to be associated with bad prognosis. The anti-CCR4 compound reduced circulating $CD4^+Foxp3^+$ Tregs and $CCR4^+$ Tregs, furthermore, the number of local CCR4 cells was reduced. The combined treatment with piroxicam better reduced the tumor size than piroxicam alone. The median progression-free survival time (PFS) was 204 (21–573) days and 57 (6–210) days in mogamulizumab/piroxicam dogs and piroxicam dogs, respectively; the respective OS time was 312 (86–1000) days and 99 (6–468) days. Observed clinical side effects were grade 1 or 2 (vomiting, anorexia, pancreatitis, urticaria, rash, and infusion reaction).

Modern studies investigate molecular targets like tight junction proteins. A recent in vitro approach used prostate adenocarcinoma (PAC) and transitional cell carcinoma (TCC) cell lines to investigate whether it is possible to destroy tumor cells by gold-nanoparticle-mediated laser perforation (GNOME-LP [143]), a noninvasive thermotherapy. The gold-nanoparticles (AuNPs) were conjugated to *Clostridium perfringens* enterotoxin (C-CPE); the latter are known to bind to claudins, which are tight junction proteins frequently expressed in tumors. They are of interest since they regulate the transfer of molecules through tight junctions and in case of deregulation because cancer might contribute to metastase spreading [144]. The targeted AuNPs enter the tumor and the laser activation leads to protein thermodenaturation. The successful laser perforation was recognized by red fluorescence signals. When the combination of functionalized AuNPs and GNOME-LP was used, cell survival was significantly reduced in comparison to non-treated control cells. The targeted treatment is a promising new approach.

In human medicine, *BRAF* inhibitors have been developed for targeted treatment of *BRAF* mutant tumors [145]; respective investigations concerning prostate cancer cell lines are ongoing in veterinary medicine [131].

Another interesting method is the prostate artery chemoembolization, causing necrosis of prostate gland and tumor tissue and a decrease in prostate volume of approximately 70% in one study [146]. The method is promising; however, since all dogs died because of metastases within 9 months, improvement of early diagnosis of the disease is most important.

4.5.6 Prognosis

The prognosis of maligne prostate cancer is poor; the median survival time (MST) is still 0–6.9 months and better in case of intracapsular growth and early-stage cancer [15, 115].

5. Conclusions

Diseases of the prostate gland are frequent disorders of the aging dogs. The symptoms sometimes are unspecific; however, in case of urination and defecation problems in older male dogs, the enlarged prostate gland must be considered. The andrological examination must include the whole urogenital tract. The Benign Prostate Gland Hyperplasia (BPH) develops slowly and mild symptoms like bloody preputial discharge are typical at the beginning. Using routine diagnostic pathways, starting with a thorough case history followed by clinical examination including digital-rectal palpation and B-mode sonography, the correct diagnosis is quickly made in most cases. Measurement of the CPSE serum concentration can be done; however, the result must be carefully interpreted, considering the age of the dog. When the well-being of the dog is disturbed or sonography of the prostate gland reveals signs of a chronical inflammation, further examinations are necessary. Semen collection with cytological and bacteriological examination of the sperm-rich fraction or prostatic secretion is one possibility. If this is not possible, transcutaneous puncture of cysts can be performed, eventually followed by FNA or biopsy of the diseased tissue. All samples should be examined cytologically and bacteriologically; cytological findings well correlate with FNA findings, and the bacteriological examination should always be combined with a resistance test, since antibiotics in chronical cases, have to be applied for weeks. Prostate gland tumors can only be diagnosed by FNA or biopsy; the search for reliable biological markers and new imaging methods is ongoing. New therapeutical methods such as immunotherapy combined with NSAIDs, targeted noninvasive thermotherapy, *BRAF* gene inhibitors or prostate artery chemoembolization are currently under investigation.

Conflict of interest

The author declares no conflict of interest

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Chapter 7

Canine Hearing Management

Peter M. Skip Scheifele, Devan Marshall, Stephen Lee, Paul Reid, Thomas McCreery and David Byrne

Abstract

The United States military employs multipurpose canines as force multipliers. A newly developed baseline audiology program applicable to noise effects on the hearing threshold for these dogs has just been developed by the University of Cincinnati FETCHLAB using brainstem auditory evoked potentials to detect estimated threshold shifts in this population. Dogs that are routinely deployed are subject to consistent exposure to noise in the field. Few investigations have focused on the effects of transport noise on the auditory system in multipurpose dogs. The consequence of these dogs having a significant hearing threshold shift is a failure of the dog to properly respond to voice commands and to miss critical acoustic cues while on target. This chapter specifically discusses the baseline protocol for audiological testing of special operations' multipurpose canines related to helicopter transport.

Keywords: hearing threshold shift, hearing loss, hearing protection

1. Introduction

The United States Military employs multipurpose canines as force multipliers. The primary breeds of dogs serving are the Belgian Malinois and the German Shepherds. A baseline audiology program has previously never been developed that is adequate to their needs as it applies to noise effects on canine hearing. Thus, there remains a need for criteria to be developed for canine auditory fitness. Presently, auditory fitness in dogs is judged by handler observation of canine behavior, including response to verbal commands, veterinary otoscopic examination, and ability to train [1].

Constant noise can have physiologic and psychological effects in several nonhuman species [2]. This investigation was focused specifically on the deleterious effects of environmental noise on the auditory system in dogs. Whether constant noise can affect dogs, particularly working dogs that are relied upon for their enhanced sensory capabilities (e.g., those used in special military operations or search and rescue), it is important to determine the conditions or environments that can potentially impair these sensory capabilities to adequately understand their impact on canine hearing. The most important frequencies for multipurpose canines to hear in practicality are in the human audible range of 20–20,000 Hz (even though dogs are very sensitive to higher frequencies past 20,000 Hz) since, operationally, it is paramount for the dogs to be able to take vocal commands from the handler and that higher frequencies

attenuate rapidly in the field. This requirement is based on handler and veterinarian requests for the information (personal contact, unpublished).

Although, anatomically, the canine ear canal differs from humans and the canine cochlea differs anatomically (where dogs have a higher range of frequencies of hearing than humans), functional magnetic resonance imaging (fMRI) studies have shown analogies between human and canine auditory cortices and central auditory systems [1–4].

As a result of the number of cases of congenital deafness in dogs, the veterinary and breeding communities have made an extensive effort to perform auditory screening between the ages of 5–8 weeks of age. The only acceptable audiological test for determining baseline hearing acuity is the brainstem auditory evoked response (BAER) test [5–12]. We are using BAER testing for threshold estimation as a baseline for establishing current hearing threshold in dogs in the current protocol. Another test that can be used for baseline and routine follow-up testing is the distortion product otoacoustic emission (DPOAE) [10, 11, 13]. In addition, the auditory steady-state response (ASSR) has also been used to evaluate hearing in dogs [10] The Malinois breed is not one of those recorded on the list of breeds known to suffer from congenital deafness, although the German Shepard dog is on the list [14].

The BAER electrophysiological test is relatively objective in its output (waveforms); however, the establishment of which peak on the resultant waveforms is subjective with the possible exception of Wave-V and the subsequent trough (VT) of Wave-V. This routine technique that has been used with humans since 1967 [15] and slowly introduced into the animal industry since the 1980's [5–7, 11, 13].

The comparison of evoked responses with behavioral hearing thresholds would be the norm when attempting to determine the normal hearing threshold of an animal [1]. In this situation, the subjects of this testing were dogs that were already kenneled for some time and had already been in previous flight training situations. The testing was conducted using an opportune time when flight training was underway.

Figure 1 shows the canine hearing threshold. **Figure 2** is an example of a typical canine BAER waveform. **Figure 3** shows hearing thresholds for tested subjects. **Figure 4** shows examples of the BAER waveforms for a tested subject. Outside of congenital deafness, elevated hearing thresholds have been recorded in military working dogs (MWDs) during transport in trucks and helicopters, when exposed to gunfire and explosives, and commonly in working dog kennels (data from samples taken on military bases-unpublished).

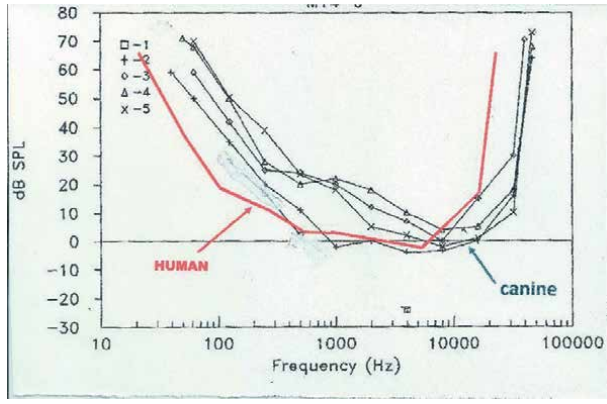

Figure 1.
These are behavioral hearing threshold curves for various breeds of dogs [16], where 1 is generalized canine threshold, 2 is Poodle, 3 is Dachshund, 4 is Saint Bernard, and 5 is Chihuahua.

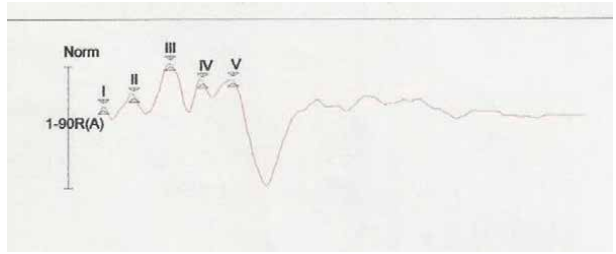

Figure 2.
A typical canine BAER trace taken in the right ear of a dog at 90 dB peSPL (54 dB nHL) using a broadband 100-microsecond click stimulus. Various peaks are marked as stops along the auditory pathway.

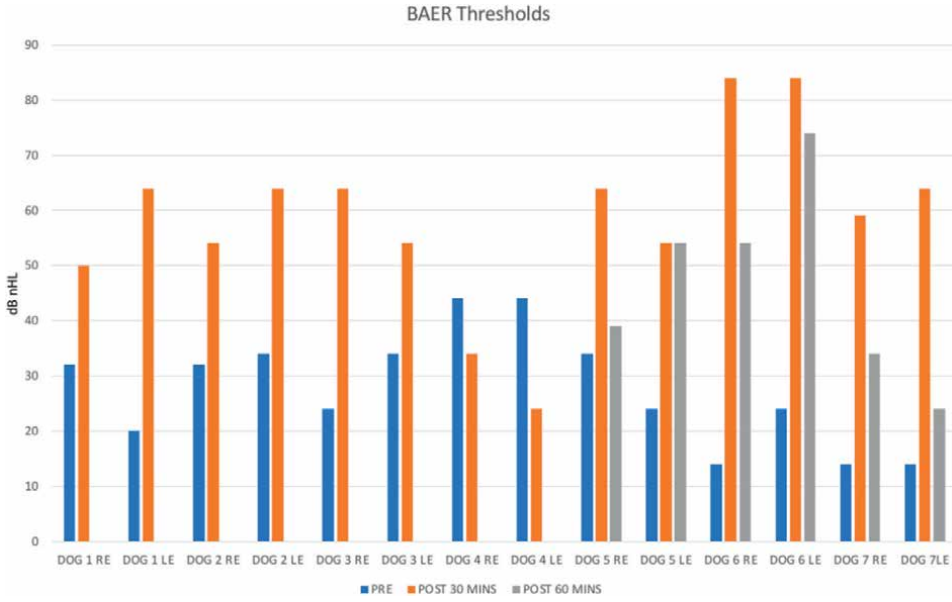

Figure 3.
Hearing thresholds based on BAER tests to estimate threshold shifts obtained pre- and post-30-minute and 60-minute military helicopter flights for seven multipurpose canines using a 100-microsecond click stimulus over ER-3 ear inserts.

Most occupied military kennels may have peak noise at 100 dBA, which requires hearing protection of the handlers upon entering [17–20]. The consequence of significant elevated thresholds is a failure of the dog to properly respond to voice commands and to miss critical acoustic cues while working, especially when working in gunshot or explosive noise (Personal correspondence) (**Table 1**).

Routinely deployed dogs are subject to relatively consistent exposure to noise in the field during training and operations. Although hearing protection devices (HPDs) for canines exist commercially, those that we have tested do not sufficiently attenuate frequencies below 1000 Hz. These low frequencies are particularly important to attenuate for multipurpose dogs that are exposed to machinery, helicopter flights, certain military operations, and explosives [21–23]. Currently, when multipurpose canines are transported in helicopters, each handler uses his/her own method for ear protection, and in many cases, no hearing protection device is used. The Army Research Office has awarded tasking to develop both over-the-ear (snood) and in-ear

electronic HPDs. The dogs tested in this project were undergoing routine flight training and were not wearing any hearing protection except for one dog who wore a snood to see what BAER thresholds would result in after using an HPD.

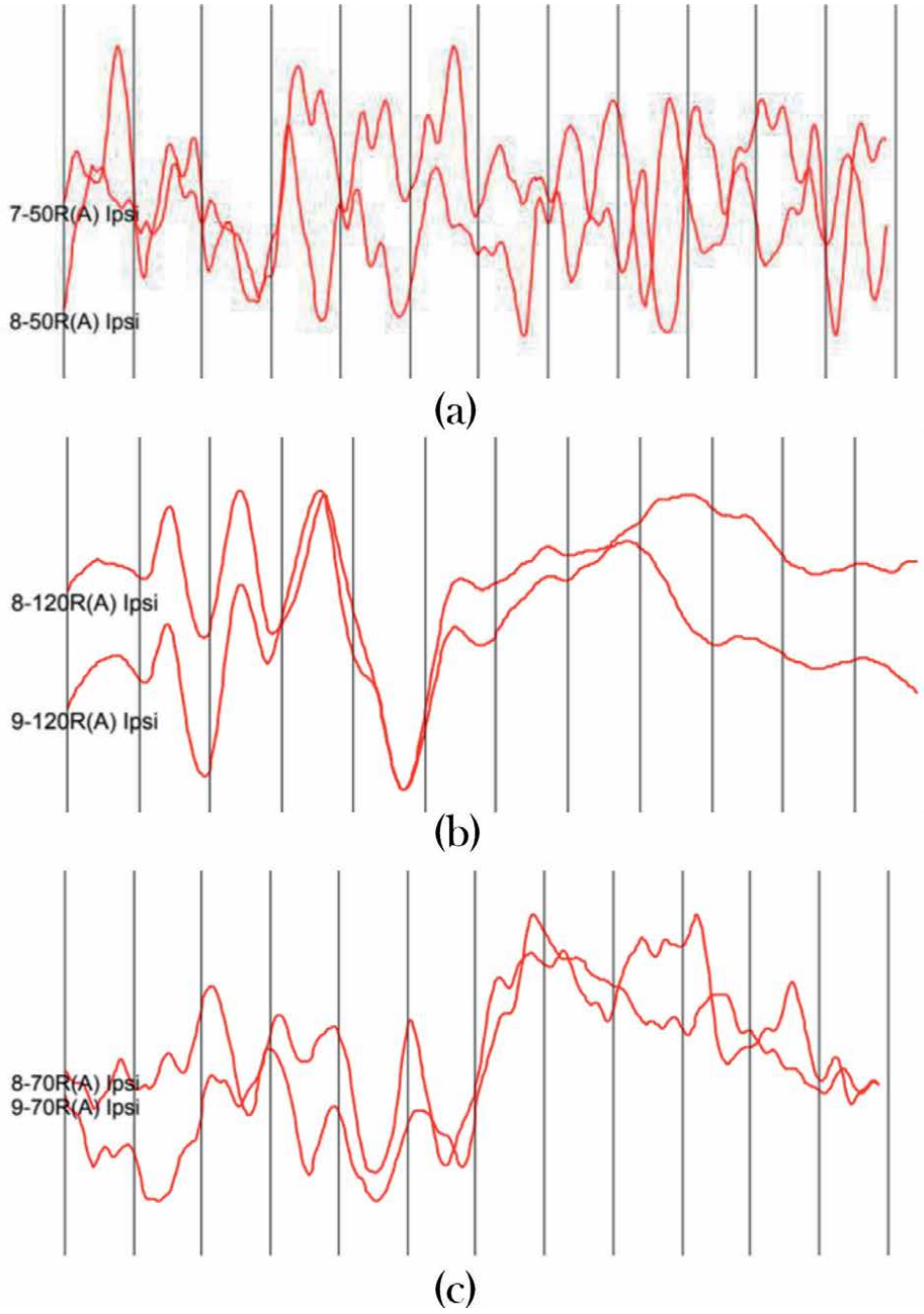

Figure 4.
Example of a (A) baseline BAER threshold at 50 dB peSPL, (B) 30-minute postflight BAER threshold at 120 dB peSPL, and (C) 60-minute BAER threshold after helicopter flight noise exposure at 70 dB peSPL showing threshold recovery over time.

	Dog 1 RE	Dog 1 LE	Dog 2 RE	Dog 2 LE	Dog 3 RE	Dog 3 LE	Dog 4 RE	Dog 4 LE	Dog 5 RE	Dog 5 LE	Dog 6 RE	Dog 6 LE	Dog 7 RE	Dog 7LE	Average
Preflight	32	20	32	34	24	34	44	44	34	24	14	24	14	14	27.71
Post 30 minutes	50	64	54	64	64	54	34	24	64	54	84	84	59	64	58.35
Post 60 minutes									39	54	54	74	34	24	46.5

Table 1.
Descriptive statistics for all dog hearing thresholds in units of dB HL.

This chapter will specifically discuss our newly accepted baseline protocol to be used for the audiological testing of military working dogs and the impacts of helicopter transport noise as an example on working canine hearing threshold. This protocol has now been accepted by the Army Special Operations Command and is in use at multiple bases where multipurpose canines are trained and housed.

2. Methods

The BAER test provides an electrophysiological measure of neural responses (from the cranial nerve VIII and lower brainstem auditory nuclei) to auditory stimuli through the use of surface or subdermal electrodes. The technique is a widely used objective measure of auditory system function in humans and has also been used extensively in the auditory assessment of dogs [4].

Typically, a BAER test involves five waves occurring within 6–15 milliseconds following the evoking stimulus [3]. The fourth and fifth waves in the sequence will, occasionally merge into a single broad wave or a wave IV/V complex making identification of all the five waves difficult to define. Our experience with puppy screening and diagnostic testing in the FETCHLAB clinics is that we do see five waves most of the time. The second wave in the sequence (wave Il) is often of sufficiently small amplitude that it is masked by the background recording noise and, therefore, not readily identifiable [7, 8]. These variances in the morphology of BAER recordings are not considered unusual and likely result from an interaction between the selected electrode placement sites, acquisition parameters, and electrical transmission characteristics of the various tissues interposed between the neural generators and the electrode recording site [8].

When BAER testing is used to quantify hearing threshold levels, the most commonly used interpretation metric involves the identification of the lowest stimulus intensity at which the fifth peak in the sequence, or wave V, can be identified (the wave V threshold). This is also known as the lowest observable response level (LORL) [17].

3. Baseline test procedure

This testing was accomplished under the University of Cincinnati IACUC protocol #07-12-19-01 and USAMRMC proposal number 18263008, award number A2-7467. This protocol was approved after annual review on July 18, 2021. The hearing evaluation was considered normal clinical testing. Moreover, advantage was taken of these multipurpose canines undergoing normal flight (helicopter) training. We simply conducted pre- and posthearing evaluations to obtain auditory data for this research. Given the limitations placed on us to have access to these dogs, we were only able to conduct limited further threshold BAER testing at later times postflight to observe changes in the postflight threshold shifts.

Seven [7] military working dogs (MWDs) ranging from 2 to 5 years of age were baseline tested at a military base veterinary clinic using the following procedure: a BAER threshold estimation test was run using a 100-microsecond click stimulus on an Intelligent Hearing Systems (IHS) unit. This test was conducted using the following parameters:

Polarity: rarefaction.

Rate: 31.1.

Sweeps: 500.

Stimulus intensities: 110 dB peSPL (74 dB nHL), 100 dB peSPL (64 dB nHL), 90 dB peSPL (54 dB nHL), 80 dB peSPL (44 dB nHL), 70 dB peSPL (34 dB nHL), 60 dB peSPL (24 dB nHL), 50 dB peSPL (14 dB nHL).

*There is a 34-dB conversation/calibration factor from dB peal sound pressure level (peSPL) to dB normal hearing level (nHL) on the particular IHS system used for this project.

Amplification: 100,000.

Low-pass filter: 1500 Hz.

High-pass filter: 100 Hz.

Stimulus: 100 microsecond click.

Based on the results of the baseline BAER, a Wave I–V latency intensity function was developed for each ear.

The BAER test analysis consisted of observing similar wave 1, III, and V latencies in two separate runs.

All the dogs were taken directly from their kennel and then pretested in the veterinary clinic in the kennel complex. Prior to testing, an otoscopic examination was conducted to ensure that no occlusion or the possibility that conductive issues were present. The tympanic membrane was viewed in all the dogs. The dogs then proceeded directly to their flight training where they were flown for 30 minutes in an H60 helicopter as usual. The handlers normally used their own means of protection for the dog such as simply folding the ears over, cotton, or nothing at all. In this case, the handlers elected not to use any form of hearing protection with the exception of one handler by choice. This flight time was shorter than normal although, depending on the mission, helicopter transit times vary greatly. At the end of flight, they were immediately brought back to the veterinary clinic and retested (postflight). Threshold estimations were noted. Dogs were sedated during BAER testing procedures with dexmedetomidine based off weight to minimize muscle artifact.

The procedure began with BAER testing each dog at an intensity of 76 dB hearing loss (HL) and increasing the stimulus intensity by 10 dB HL until all the waves were present and a second set of similar waveforms existed. Then, the intensity was lowered by 5 dB HL until similarity was no longer present. This, then, constituted the dog's threshold for that test sequence. The same protocol was followed postflight, 30 and 60 minutes later.

4. Noise level in flight

The noise level within the helicopter was measured using a Bruel and Kjaer model 2270 sound level meter. The average noise level (LAeq) was measured over the entire 30-minute flight. An example LAeq from a 30-minute helicopter flight in this project was 107 dBA. An A-weighted decibel (dbA) is a scale for measuring loudness corresponding to hearing thresholds of the human ear.

5. Results

After 30-minute in-flight, a 30-minute postflight BAER threshold estimation was run upon touchdown and with a retest 60-minute postflight to estimate threshold shifts. Results were compared to baseline thresholds. A total of seven canines (all male)

were included in this study. Decibels (dB) are defined in different reference units. The dB peSPL is a decibel in sound pressure level comparing the pressure of sound at the microphone of the sound level meter to the reference pressure of 0.0002n dynes/cm^2. The results are listed in dB nHL, which is a decibel in normalized hearing level when using electrophysiologic testing, such as a BAER test. A conversation factor of 36 dB can change the dB SPL value to dB nHL with the particular IHS machine used for this study. The average threshold of baseline BAER thresholds was 27.71 dB nHL, the average of 30-minute postflight BAER thresholds was 58.35 dB HL, and the average of 60-minute postflight BAER thresholds was 46.5 dB HL. BAER thresholds increased by an average of 30.64 dB HL after 30 minutes postflight, which is equivalent to a moderate-to-severe hearing loss. BAER thresholds decreased after 60 minutes postflight by 11.85 dB HL, which is equivalent to a mild hearing loss. Dog 4 used a Zeteo Tech, Inc. snood-type canine auditory protection system (CAPS) for hearing protection, which resulted in having better thresholds 30 minutes postflight than the baseline. Dog 4 was subjected to kennel noise before baseline testing and had suspected elevated thresholds greater than expected.

6. Discussion

It has been shown that threshold shifts can occur in humans following exposure to noise levels between 90 and 125 dB SPL (8). A temporary threshold shift (TTS) may include a temporary reduction in hearing acuity, which may become evident within minutes after exposure but is usually reversible in time (this time is variable across individuals).

The underlying pathophysiologic changes involve cell death among various sensory and support cells in the inner ear, resulting from an oxidative stress reaction due to long-term overstimulation [15]. Given the similarities of the typical mammalian auditory system and specifically the canine versus human auditory systems, it is not unreasonable to imply that the triggering causes and attributes of the noise-induced hearing loss would be similar if not identical to that of canines.

From a behavioral perspective, canine handlers reported that after touchdown during mission helicopter flights, their canines were not reacting to standard verbal commands, reducing the tactical effectiveness of the canines. Handlers reported that it appeared that the canine was either not listening, was seemingly disoriented, or not readily responding to verbal commands for various lengths of time, which seemed to be canine specific (personal communication). The dogs in this flight training did not show any disorientation but did not react in the usual manner to vocal command once exiting the helicopter. The flight time during this training was shorter than most mission flights. Given the average threshold shift shown by the test dogs in this study (approximately 30 dB nHL), it is reasonable to presuppose that the threshold shift played a significant role in this behavior.

Presently, the actual prevalence of hearing loss in MWDs is unreported or classified. Given the newly established baseline auditory testing for MWDs, these statistics will become available, thus allowing for longer service and care of the dogs.

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Edited by Carlos Eduardo Fonseca-Alves

Veterinary medicine has grown in importance in recent years as dogs have become even more important in human society. For many years, dogs have served as protectors, companions, and even workers. The close relationship between dogs and humans has created a need for specific techniques and interventions for their care. Advances in canine medicine have resulted in dogs living longer, healthier lives. However, dogs are still susceptible to diseases and illnesses. This book provides a comprehensive overview of canine medicine and advances in the diagnosis and treatment of infectious, degenerative, and immunological diseases, cancer, and much more.

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