Airway Deposition of Nebulized Voriconazole in Horses

by

Tamara Sierra Rodriguez

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
December 12, 2020

Keywords: equine, voriconazole, nebulization, pneumomycosis,
fungal pneumonia, fungal rhinitis

Approved by

Anne A. Wooldridge, Chair, Professor, Department of Clinical Sciences
Kara M. Lascola, Associate Professor, Department of Clinical Sciences
Sue H. Duran, Professor, Department of Clinical Sciences
Erin S. Groover, Associate Clinical Professor, Department of Clinical Sciences

Abstract

Voriconazole (VRC) is a potential treatment for pneumomycosis in horses. The objectives of this study were to determine if delivery of Vfend® using a Flexineb® nebulizer produced clinically significant [VRC] in lower airways. The hypothesis was that [VRC] after delivery by nebulization would be higher in the pulmonary epithelial lining fluid than plasma. A secondary objective was to determine [VRC] in upper airways through collection of nasopharyngeal wash (NPW) samples.

Voriconazole solution (Vfend®-6.25 mg/mL, 100 (n=2), 200 (n=3), 500 (n=1) mg) was nebulized once in 6 healthy geldings. Clinical responses, duration of nebulization, and [VRC] at various timepoints (up to 8 hours) in plasma, bronchoalveolar lavage fluid (BALF) supernatant and cell pellet, and NPW samples were recorded. Voriconazole (Vfend®-6.25 mg/mL, 200 mg) was nebulized in 5 additional, healthy geldings, and [VRC] was measured in NPW samples pre- and post-nebulization at timepoints up to 8 hours. Antifungal activity of BALF and NPW samples was determined using agar disk diffusion.

Concentrations of voriconazole were below detection in plasma, BALF supernatant and cell pellets for all timepoints and doses except the BALF cell pellet (0.4 µg/g) immediately after nebulization of 500 mg. For 5 horses administered 200 mg of Vfend®, mean [VCR] in NPW at the end of nebulization and 1, 6, and 8 hours post-nebulization were: 30.8 ± 29, 1.0 ± 0.84, 0.2 ± 0.19, and 0.34 ± 0.67 µg/mL, respectively. Only NPW samples obtained immediately post-nebulization showed antifungal activity.

Nebulized Vfend® solution is not recommended for treatment of pneumomycosis in horses.

Acknowledgments

First, I would like to acknowledge the members of my committee for their mentorship and assistance to design and execute this project. Dr. Anne A. Wooldridge, my principal mentor for this project, has been an invaluable teacher and assistant to design and execute this project. She has been an incredible support and mentor to me during the residency program. She has taught me how to design a research project, how to write grant proposals and scientific articles, and has been a key person in my formation as a clinician and researcher. I would also like to thank my co-investigators and mentors. Dr. Erin S. Groover has also been vital in helping me to design and execute this research project and has been my principal mentor in the residency training. She has been a key component in this journey, and I thank her for her endless support and patience to teach me and make me a better internist. Dr. Kara Lascola has also been a great support and mentor for my research and an important part of my professional training. I also want to thank Dr. Sue Duran for her willingness to help me brainstorm about this project and always being available if I had any questions. She has been a great mentor and I thank her for everything she taught me about pharmacology.

I would also like to acknowledge the help of Dr. William Ravis and Dr. Phillip Lee, as this project would not have been possible without them. Drs. Mariano Mora and Elizabeth Spangler, and Terri Hathcock, I also thank you for your invaluable mentoring and support with this project. Dr. Mora has not only been helping me with this project but has also been a great mentor and friend in my residency training and I am truly thankful for his patience and mentoring. Finally, I would like to acknowledge Jessica Brown's technical support to execute this study and helping me care for the horses involved in the study.

Moreover, and most importantly, none of this would have been possible without the help and support from my family, boyfriend, and friends. I want to thank every person that encouraged me to pursue this journey and to those that gave me the opportunity to fulfill one of my biggest dreams as a veterinarian.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar fluid
BALT	Bronchial-associated lymphoid tissue
DNA	Deoxyribonucleic acid
HPLC	High performance liquid chromatography
IPA	Invasive pulmonary aspergillosis
IV	Intravenous
LRT	Lower respiratory tract
MIC	Minimum inhibitory concentration
MMAD	Mean mass aerodynamic diameter
NPW	Nasopharyngeal wash
PCR	Polymerase chain reaction
PELF	Pulmonary epithelial lining fluid
pMDIs	Pressurized metered dose inhalers
TW	Tracheal wash
URT	Upper respiratory tract
VRC	Voriconazole

Chapter 1 - Literature review

1.1 Equine Respiratory Tract Anatomy and Physiology

The respiratory system is responsible for delivering oxygen to and removing carbon dioxide from the blood[1]. It is divided into the upper respiratory tract[2] (URT) and lower respiratory tract (LRT).

The URT starts with the nasal cavity, which is separated by the nasal septum and is covered with a highly vascular mucosa. The rostral end of the nasal mucosa is covered by a non-ciliated stratified cuboidal epithelium with a low density of goblet cells and continues caudally with a ciliated pseudostratified columnar epithelium that progressively increases in goblet cell density[3]. Each side of the horse's nasal cavity has two turbinates that divide the cavity into 3 air passages, the ventral, middle and dorsal meatuses. The turbinates enlarge the mucosal surface of the nasal cavity to facilitate the air-conditioning and defense functions. The turbinates scroll to form the nasal conchae, which are contiguous with the frontal and maxillary sinuses.

The paranasal sinuses consist of seven pairs in the horse, which are the rostral and caudal maxillary, ventral and dorsal conchal, frontal, sphenopalatine, and ethmoid sinuses[1]. Postganglionic sympathetic nerves, which are supplied via the cervical sympathetic preganglionic fibers synapsing in the superior cervical ganglion, innervate the nasal blood vessels causing vasoconstriction. Parasympathetic innervation of the nose is from the facial nerve and has little effect on the diameter of blood vessels, but regulates glandular blood flow and secretion. The axons of nerves in the olfactory epithelium converge to form the olfactory cranial nerve giving rise to the sense of smell.

The pharynx delivers air from the posterior nasal cavity to the larynx. In horses, the oral cavity and pharynx are normally separated by the soft palate except during swallowing. For this reason, horses are obligate nasal breathers[1]. The nasopharynx is lined with pseudostratified columnar ciliated epithelium containing goblet cells, and the oropharynx is lined by stratified squamous epithelium. Lymphoid tissue that is organized into follicles is visible in rows along the dorsal wall and represent part of the equine tonsil. The guttural pouches, which are only present in Equids, are paired ventral diverticuli of the Eustachian tubes that extend from the nasopharynx to the middle ear. They are lined by ciliated epithelium with mucus-secreting cells and have a close association with the vagus, glossopharyngeal, and hypoglossal nerves. The internal carotid artery runs in the wall of the guttural pouch, and it is thought that this association allows for cooling of arterial blood on its way to the brain[4].

The larynx has the main function of preventing inhalation of food into the lower airway during swallowing, and phonation as a secondary function. It has a cartilaginous support, which is provided by the ring-shaped cricoid cartilage adjacent to the first tracheal ring, the large thyroid cartilage, a pair of arytenoid cartilages that support the vocal folds, and the epiglottis that provides a protective flap to cover the glottis during deglutition. The corniculate and cuneiform cartilages are attached to the arytenoid and epiglottic cartilages, respectively. Innervation of the intrinsic muscles of the larynx is via the recurrent laryngeal nerves except for the cricothyroideus, which receives innervation via the cranial laryngeal nerves. The laryngeal mucosa is contiguous with that of the pharynx and trachea. Between the lateral border of the epiglottis and the cuneiform/arytenoid cartilages, the mucosa forms the aryepiglottic fold. Most of the larynx is lined by respiratory

pseudostratified, columnar, ciliated epithelium with goblet cells, but the epiglottis and vocal folds are covered by a stratified squamous epithelium. There are numerous mucus glands beneath the laryngeal mucosa, especially in the epiglottis[1].

The LRT starts with the trachea, which is supported throughout its length by C-shaped sections of cartilage that are tightly opposed to one another to give rigidity to the airway. The pseudostratified columnar tracheal epithelium consists primarily of mucus-producing goblet cells and ciliated cells above a layer of basal cells. The horse has few submucosal bronchial glands. The lamina propria contains many branches of the bronchial circulation that serve to warm and humidify the air and participate in the inflammatory response of the trachea. The lamina propria is richly supplied with both sensory and autonomic nerves. Non-myelinated sensory nerves containing neuropeptides ramify into the epithelium, and parasympathetic nerves reach the cranial and caudal trachea via the cranial laryngeal and vagus nerves, respectively. Postganglionic sympathetic fibers enter the wall of the trachea in association with the vagus nerve and terminate in the lamina propria around the bronchial blood vessels. Sympathetic nerves innervate the trachealis muscle only in its cranial third[5].

At the end of the trachea, the carina gives opening to the main bronchi. Each main bronchus continues almost directly toward the lung periphery and forms a series of smaller branches. Bronchi are identified by the presence of cartilage in their walls and include all airways greater than approximately 2-mm diameter. The trachea and bronchi are lined by a pseudostratified columnar epithelium that overlies the basement membrane and consists of ciliated and non-ciliated cells that differentiate from basal cells. The non-ciliated cells are primarily goblet cells. The

mucus-secreting goblet-cells produce the mucin, which form a large percentage of the mucoid layer that lines the airways. This mucoid layer is propelled cranially by the ciliary cells. The lamina propria is immediately beneath the basement membrane and contains a rich supply of bronchial blood vessels and nerves. The nerves include non-myelinated neuropeptide-containing sensory nerves and branches of the sympathetic nervous system that supply the bronchial blood vessels. Smooth muscle encircles the bronchi and bronchioles and receives parasympathetic and inhibitory non-adrenergic non-cholinergic innervation[5,6].

The bronchi are surrounded by the lung parenchyma. Unlike in other mammals, the horse lung is not divided into distinct lobes. The right lung is larger than the left because it includes the intermediate or accessory lobe. The surface of the horse lung shows the fibrous connective tissue septa that divide the lung into lobules. The separation of the lung into lobules limits the collateral movement of air between different lung regions. The greatest bulk of the horse lung is in its caudodorsal region. The bronchioles can be differentiated from bronchi by the absence of cartilage in their walls. They connect the small bronchi to the alveolar ducts and alveoli. In the bronchioles, the epithelium is a single layer of cuboidal cells. The primary secretory cell is the Clara cell that has an extensive network of smooth endoplasmic reticulum. Ciliated epithelial cells are also present in the bronchioles but are less dense in number than in the larger airways. Mucus-secreting goblet cells are not present in the bronchioles of young healthy horses but are found in horses that have airway inflammation. As in the bronchi, a layer of smooth muscle encircles the bronchioles[1]. Compared to other mammals, horses lack well-developed respiratory bronchioles, instead, the terminal non-respiratory bronchiole connects directly to the alveolar duct[7].

The gas exchange occurs in the alveolar ducts and alveoli. The former are extensions of the bronchioles, each of which has numerous alveoli in its walls. Two types of epithelial cells line the alveoli. The type I cell, which covers most of the surface, is characterized by very thin cytoplasmic extensions that extend away from the nucleus over the alveolar surface. Its cytoplasm has few organelles other than pinocytotic vesicles and a few mitochondria. In comparison, the type II cell shows evidence of being metabolically very active. Its cytoplasm is rich in endoplasmic reticulum and the Golgi apparatus is large. The type II cell has large vesicles that contain the precursors of pulmonary surfactant, the phospholipid that is essential for lung stability. Surfactant is released from the type II cells in the form of myelin coils that unfurl when they reach the alveolar surface. Type II cells also re-uptake components of surfactant to be resynthesized. In addition, these cells also reabsorb edema fluid from the alveoli, and, when the alveolar surface is injured and type I cells are lost, the type II cells differentiate into type I cells to recover the surface.

The alveoli are separated from one another by the alveolar septum that contains the pulmonary capillaries. The air is separated from the capillary blood by the type I epithelial cell, a basement membrane, a variable amount of interstitium, and the endothelial cell. The alveolar-capillary barrier is a semipermeable membrane between the pulmonary capillaries and the alveolar cavity that limits the free movement of ions, fluid and a variety of large molecules. The main type of cell junction that forms this semipermeable membrane are the tight junctions (or occluding junctions). These tight junctions are a type of cellular connection located at the apical region of pulmonary capillary endothelial cells and epithelial cells and form the closest contact between adjacent cells, thus limiting solute diffusion through intercellular spaces. Their integrity is important to maintain pulmonary vascular permeability[8]. Occludin, a transmembrane protein, and

zona occludens 1 (ZO-1), a cytoplasmic protein, are the main components of tight junctions. The downregulation or redistribution of these proteins increases the permeability between endothelial cells and epithelial cells[9]. The capillary network in the alveolar septum is very extensive. Because there are no lymphatics in the alveolar septum, fluid that filters from the pulmonary capillaries must track through the interstitium to the lymphatics in the peribronchial tissue. The lung possesses two networks of lymphatics, one that surrounds the bronchi while the other is subpleural. Both these networks connect to the hilar and mediastinal lymph nodes and drain into the thoracic duct.

The pulmonary circulation receives the whole output of the right ventricle and delivers it through the pulmonary capillaries and back to the left atrium and is involved in the uptake of oxygen and removal of carbon dioxide. The bronchial circulation is a branch of the systemic circulation from which it receives about 2% of the cardiac output. It provides the nutritional blood flow to the walls of the bronchi and large blood vessels and to the pleura. In the bronchi, the submucosal plexus of bronchial vessels is important for warming and humidifying air and in the immune response. The venous drainage of the bronchial circulation is complex. Some returns to the azygous vein but some also enters the pulmonary veins thereby adding venous blood to the oxygenated blood that is leaving the capillaries. If the blood supplied by the pulmonary circulation becomes reduced in a region of the lung, the connections between the pulmonary and bronchial circulations allow blood to enter that region from the bronchial circulation thereby tending to reduce the chance of ischemia. The bronchial circulation is involved in inflammation healing and remodeling of the lung.

The respiratory tract has different mechanisms of protection, such as non-specific, generalized, and specialized mechanisms[10]. Non-specific protection is offered by anatomic barriers, mucosal lining, mucus secretion, and the mucociliary escalator. Turbulence created in the airstream and the anatomic organization of the upper respiratory tract removes particles as small as 5 μm, thus only particles smaller than this dimension reach the alveoli. When the particles reach the trachea, the mucociliary escalator mechanism will offer protection through trapping, cough reflex, and expulsion of material from the airways. This mechanism is highly effective based on the composition of the double layer of mucus that extends from the pharynx to the bronchioles. The particle-laden mucus is swallowed and presumably digested in the intestinal tract. The goblet cells found in the upper respiratory tract mucosa secrete mucus, which effectively adsorbs soluble host molecules such as host defensin peptides, lactoferrin, lysozyme, and surfactant proteins. Microbial organisms are rapidly destroyed by this barrier. The ones that reach the alveoli are cleared via phagocytosis by alveolar macrophages.

The specific pulmonary clearance mechanisms are found within the bronchial-associated lymphoid tissue (BALT), which is localized within the submucosa of the segmental bronchi and terminal bronchioles[11]. The BALT is an area where antigen-specific responses stimulate cell-mediated and humoral immune defense. B lymphocytes within BALT can switch to all classes of antibodies, yet the predominant antibody produced in the URT is immunoglobulin-A; immunoglobulin-G is secreted mainly in the lower airways. The secretion of immunoglobulin-A in the upper airways blocks the adherence of pathogens to the URT epithelium, which is referred to as immune exclusion.

Cellular responses are also critical for immune protection of the host. The alveolar macrophages are the first phagocyte of importance and are in the terminal bronchioles and alveoli. These cells can nonspecifically ingest foreign material and serve as important antigen-presenting cells for T lymphocytes and the development of adaptive immunity. Once alveolar macrophages ingest the foreign material, they may be coughed up and swallowed or they may move from the alveolar space and enter general circulation, resulting in clearance by the lymphatic system. Host health status can greatly affect the function of these cells; viral infections and long distance transport will destroy these cells[11]. The pulmonary intravascular macrophage is another important cell for removal of bacteria or toxins from general circulation and contributes to the systemic inflammatory response that is induced following pathogen challenge. Horses, pigs, ruminants, and cats are some of the mammals that have these cells; those species that do not have pulmonary intravascular macrophages utilize hepatic Kupffer's cells and splenic macrophages for similar purposes.

Even though the respiratory tract is considered a relatively sterile environment, it actually contains abundant microbiome that provides an important endogenous protection barrier. The disruption of this microbial population, damage to the normal clearance mechanisms, or mechanical epithelial damage may, however, result in altered microbial populations leading to pathogen proliferation or invasion.

1.2 Definition and Pathogenesis of Fungal Respiratory Diseases in Horses

Understanding the anatomy and mechanisms of defense of the respiratory system is necessary to comprehend the pathogenesis of fungal infections affecting the respiratory tract, how to diagnose, and the best course of treatment.

Fungi are eukaryotic organisms with a cell wall composed of three major polysaccharides: chitin, mannose, and β-1,3 and β-1,6 linked glucans[12]. Within the fungal wall, the plasma membrane contains ergosterol, which is the target of many antifungal agents. Pathogenic fungi are divided into 3 groups: multinucleate septate filamentous fungi, non-septate filamentous fungi, and yeasts. Dimorphic fungi can interchange between forms depending on environmental conditions. For example, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *Coccioides immitis* exist in yeast form in vertebrate host tissue and hyphal/mycelial form *in vitro*.

Fungi are ubiquitous in the equine environment (hay, soil, and bedding), and fungal infections have been reported in horses of all ages, breeds, and occupations. Fungal infections may affect all levels of the respiratory tract in horses but most commonly affect the nasal passages, paranasal sinuses, guttural pouches, and lungs. Some forms of the disease may be sporadic, others occur in epidemics or are enzootic[13]. Fungal infections are considered rare in horses, however, geographic variability in frequency does exist.

Pathogenic fungi can be primary pathogens, capable of infecting immunologically normal horses, or opportunistic pathogens, capable of infecting only horses that are immunocompromised. Immune suppression in horses is most commonly associated with corticosteroid treatment or severe, concurrent, unrelated disease such as colitis or neoplasia. The most important primary

fungal pathogens that can cause systemic infection include *Histoplasma capsulatum*, *Coccidioides immits*, *Blastomyces dermatitidis*, and *Paracoccidioides brasiliensis*; and the most common opportunistic infections are caused by *Candida albicans*, *Aspergillus* spp., *Cryptococcus neoformans*, *Mucor* spp., and *Pneumocystis carinii*[14,15].

From the aforementioned list of important opportunistic pathogens, *Aspergillus* species are one of the most common organisms affecting the upper and lower airways, with *Aspergillus fumigatus* frequently isolated[16]. *Aspergillus* spp. conidia are frequently inhaled, and individuals with apparently normal defense mechanisms may rarely be infected; however those that are immunocompromised are at risk for colonization by this opportunistic organism[17]. In humans, pulmonary disease caused by *Aspergillus*, mainly *Aspergillus fumigatus*, presents with a spectrum of clinical syndromes in the lung[18], with one of the most important being invasive pulmonary aspergillosis (IPA). IPA is a major cause of mortality in severely immunocompromised people. In a human retrospective study of a 14-year period, IPA increased from 17% of all mycoses found on autopsy at the beginning of the study to 60% by the end of the study[19]. On the other hand, in horses *Aspergillus fumigatus* have been implicated in secondary pulmonary infection after an episode of enterocolitis[20] and also in the pathogenesis of recurrent airway obstruction[21].

In most cases of URT fungal disease, predisposing causes are not identified. In comparison, fungal pneumonia usually occurs in immunocompromised horses, although on occasion, the normal individual may be affected. Important predisposing factor for fungal pneumonia include qualitative and quantitative granulocyte abnormalities and the presence of devitalized tissue[15].

Respiratory fungal disease is most frequently acquired via inhalation of the causative organism. Previous reports have shown that more than 90% of particles in stable air are spores of fungi or actinomycetes[22] and the concentration of respirable dust increases 6-fold during normal stable bedding down procedures[23]. After inhalation, the causative organisms can penetrate the distal airways and alveoli because of their small sporular diameter[15]. However, some cases of fungal pneumonia are thought to arise by penetration of fungi through a compromised gastrointestinal tract or open wounds[24]. Gastrointestinal inflammation results in the disruption of the epithelial barrier, allowing for translocation of fungal organisms and subsequent systemic dissemination. Hematogenous dissemination may lead to fungal deposition in the lung[10]. Among the risk factors for mycotic distribution from the gastrointestinal tract, non-steroidal anti-inflammatory drug therapy may contribute to epithelial disruption, enhancing mucosal permeability to luminal microorganisms. Dissemination of disease is an important component of disease pathogenesis; 40% of the horses with fungal pneumonia have evidence of invasive fungal disease in other organs including the kidney and brain[10].

Fungal virulence factors may be more complex than those of bacteria because of the higher degree of opportunism that occurs with a change in host status. There may be subtle factors that, combined with host status, result in a certain fungus attaining a virulent state. Cellular adherence is an important prerequisite for infection and colonization of the host. Adhesins have been identified in *Candida albicans* and *Blastomyces dermatitidis*. Two genes have been associated with adhesions in *Candida albicans*. The first is a glycoprotein that has sequences consistent with agglutinating activity. Transformation of this gene into other non-adherent fungal species results in adhesion of the transformed yeast to cells. *Candida albicans* also has integrin-like proteins, the

disruption of which results in diminished hyphal growth, adhesion to cells, and loss of virulence in mice[12].

Many fungi have polysaccharide capsules that, like bacteria, help resist phagocytosis and immune activation. For example, the capsule of *Cryptococcus neoformans* inhibits leukocyte accumulation, cytokine secretion, and macrophage phagocytosis. Mutants without capsules are highly infective and virulent. Many fungi such as *Candida albicans*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* are engulfed by macrophages, and intracellular survival is mediated by virulence factors. *Histoplasma capsulatum* is primarily a yeast in vivo, and this form infects macrophages. Fungi also secrete many degradative enzymes, including proteinases, phosphatases, and DNAses, to surmount structural barriers. A group of genes called secreted aspartyl proteinase genes allow more persistent colonization of host surfaces and deeper penetration[12].

Fungi induce apoptosis, which may be due either to the direct effect of a fungal toxin or secondary to host cell cytoskeleton rearrangements. For example, the gliotoxin of *Aspergillus fumigatus* can induce DNA fragmentation and apoptosis in macrophages. This toxin also has many other immunosuppressive qualities, which include inhibition of the neutrophil respiratory burst and T-cell activation[14].

1.3 Diagnosis and Treatment of Fungal Respiratory Diseases in Horses

The diagnosis of fungal respiratory disease is based on the history, clinical signs, diagnostic imaging, histopathology and cytology, culture, and/or molecular examination of tissue or fluid samples[15].

The clinical signs are non-specific of the disease and depend on the localization of the infection within the respiratory tract, its nature, and its chronicity. Mycotic granulomas have been found in the nasal passages, paranasal sinuses, nasopharynx, guttural pouch, trachea, bronchioles, lungs, and mediastinum of infected horses. Mycotic plaques in the guttural pouch are often associated with the arterial blood supply and may progress to cause fatal epistaxis. Pulmonary fungal infections causing granulomas, diffuse pneumonia, or pleuropneumonia can present with signs similar to bacterial infection, such as fever, tachypnea, dyspnea, nasal discharge, lethargy, etc.

Several diagnostic tests are available and are helpful to determine the etiologic pathogen. Endoscopic examination is a main diagnostic tool to diagnose infections of the respiratory system, especially those of the URT and part of the tracheobronchial tree[25]. It allows direct visualization of granulomas or fungal plaques located in the nasal passages, nasopharynx, guttural pouch, paranasal sinuses, trachea, or bronchioles. More detailed images of the equine skull can be obtained with computed tomography or magnetic resonance and helps to determine the extent of lesions and bony invasion. Another important diagnostic tool is ultrasonography, especially when evaluating the thoracic cavity, as may reveal the presence of cavitary lung lesions, lung consolidation, comet tail artifacts, or free pleural fluid. Thoracic radiographs may result in characteristic infiltrative patterns that are indicative of fungal pneumonia, such as miliary patterns or patchy bronchopneumonia with lesions most commonly affecting the periphery.

Collection of respiratory secretions is a very valuable diagnostic tool. Secretions are usually collected by tracheal wash (TW) or bronchoalveolar lavage (BAL). Cytologic and microbiologic evaluation of respiratory samples provide invaluable information about the disease process. Secretions originating from affected lung segments will eventually collect in the trachea, thus cytologic examination and microbiologic culture of TW fluid is likely to yield an etiologic diagnosis. In contrast, fluid collected by BAL is only representative of the lung region distal to the bronchus where the tube or endoscope was wedged. In general, TW is the preferred method for localized infectious cases and BAL is preferred for characterizing diffuse lung disease. When the etiology is unknown, it is best to perform both tests in order to maximize diagnostic yield with TW performed first to avoid cross contamination between procedures[26]. In cases of pleural effusion, a thoracocentesis is performed for diagnostic and therapeutic purposes, and a cytologic examination and microbiologic culture can be performed as well.

Collection of tissue samples from the respiratory tract not only is a valuable diagnostic tool but can be very useful as a prognostic indicator. Such samples allow for histopathology and for more advanced testing such as immunohistochemistry. Sampling of airway tissue can be done under bronchoscopic guidance, for endobronchial sampling, or by more invasive techniques like percutaneous or thoracoscopic lung biopsy[26].

For some fungal infections, molecular methods could be used for diagnosis. PCR is the most common method used to detect fungal DNA in samples. For example, universal primers detect virtually all species of fungi; genus-specific primers identify fungi belonging to a single genus as in detection of all *Aspergillus* spp., and species-specific primers may be used for detection

of a single species. The PCR using universal fungal primers can be helpful when the histologic examination suggests a fungal infection, including hyphae, but isolation was not successful. If a sample is positive, further testing (such as sequencing of PCR product, DNA probe) can be used to identify the genus and species present. Molecular methods for detection exist for many fungi[27]. Most of these techniques are used to detect *Aspergillus* spp., *Cryptococcus* spp., and *Candida* spp[28,29]. However, a disadvantage of using molecular methods for diagnostic testing is that fungal cells can be scarce in infected tissues, making detection difficult. These methods should not replace primary culture, isolation, and identification, but rather supplement these standard diagnostic techniques to provide the quickest and most accurate diagnosis.

Testing for fungal antigens is available to aid diagnosis of fungal infections in humans and animals. The most frequently used methods are latex agglutination and enzyme immunoassay (EIA) for detection of *Cryptococcus* spp. capsule, as these are released into the systemic circulation and allows detection of fungal antigen in serum or exudate from the site of suspected infection. Thus, because this test is a quantitative test, it can be used during treatment to evaluate response[30]. There are also other antigen-based methods to detect invasive *Aspergillus* spp. infection in humans, which most often use EIA to measure galactomannan (a component of the fungal cell wall) in body fluids or serum, however these tests have not been validated in horses to diagnose aspergillosis and even in human patients, they are controversial because cutoff values are not universally agreed on[27].

Serologic testing has been used in horses to confirm fungal infection, but its use has been limited. Serology for *Aspergillus* infection has been performed for horses using agar gel

immunodiffusion, immunoblot, or enzyme-linked immunosorbent assay (ELISA)[31]. Serology has also helped identify horses with coccidiomycosis, and although 4% of normal horses have a positive titer, a positive test result in a horse with clinical signs should still be highly suggestive of infection and can be used for prognosis[32].

Treatment of fungal respiratory disease is challenging in the horse, and prevention of fungal infections is not possible because of the ubiquitous nature of fungal spores in the environment. Currently, the most important prophylactic measures are treating predisposing illnesses promptly and effectively and judiciously avoiding overuse of corticosteroids and broad-spectrum antimicrobials. In immunocompromised patients, improving ventilation and minimizing exposure to inspired spores are most beneficial. Medical management of fungal disease in horses is hindered by not having many cost-effective, bioavailable drugs that can be effectively administered, and regardless of the medical therapy selected, it requires weeks to months of therapy for treatment benefit to be achieved[10].

Treatment of fungal granulomas of the upper respiratory tract typically involves surgical therapy alone or in combination with topical and/or systemic antifungal therapy. Excision of the lesions with or without cryotherapy performed on surgical margins after removing the affected tissue[15] are common treatments for fungal rhinitis. In cases of paranasal fungal infections, trephination of the sinus or a bone flap is required for drainage and lavage, and to apply topical antifungal medication. Mycosis of the guttural pouches almost always require surgical treatment because of the localization of the fungal plaques that tend to affect the walls of the major blood vessels found inside the pouches, and over time, the fungus can erode through the wall of the

vessels resulting in life-threatening hemorrhage. Surgical treatment consist of occluding the affected artery to eliminate the risk of hemorrhage and eliminate blood supply to the fungal plaque[33]. The use of systemic antifungal drugs to treat URT fungal infections tend not to be very efficacious, and this may be due to the drug not reaching the minimum inhibitory concentration (MIC) in the affected tissue, thus almost all cases are treated with a combination of local and systemic therapy to have the best outcome.

Treatment of mycotic pneumonia is associated with minimal success because of the rarity of early diagnosis and a concurrent severe underlying illness that is commonly present. However, successful therapy with systemic ketoconazole, amphotericin b, and voriconazole, and empirical nebulization of enilconazole had been reported for fungal pneumonia in horses[34] caused by *Scopulariopsis* spp., *Cryptococcus* spp., or *Aspergillus* spp[35-37]. Treatment of pulmonary fungal infections is currently based on the use of systemic agents alone, because there is a lack of data on aerosolized administration in horses. However, aerosolized delivery of antifungal agents is an attractive option and has been evaluated for prevention[38] and treatment[39,40] of IPA in humans[41] because the drug can concentrate locally at the site of infection with minimal systemic exposure. In horses, the use of inhaled antifungals has been only empirical and implemented in cases as a last resource[35].

A major concern when treating a fungal infection is that therapeutic options are limited and due to the difficulty identifying the etiologic agent and their drug sensitivity, choosing the most appropriate antifungal from the beginning is difficult. Luckily, fungal susceptibility testing is becoming more readily available, with the MIC for common fungal organisms against some

antifungal drugs recently determined[15]. In addition, fungal infections tend to require long-term therapy and most of the antifungals are associated with important side effects that make the treatment plan even more difficult. The selection of drug therapy should depend on the presence of complicating factors, such as organ dysfunction that might affect drug pharmacokinetics, relative drug toxicity, prior exposure to antifungal agents, and identification of organism involved and potential susceptibility pattern of the isolate[42].

The currently available antifungals target four different cell functions: pyrimidine analogs (affect DNA synthesis), echinocandins (affect cell wall integrity), polyenes (affect cell membrane integrity), and azoles (affect ergosterol biosynthesis); with the last 2 being the most commonly used in horses.

Amphotericin B, nystatin, and natamycin are polyene antifungals that combine with ergosterol in the fungal cell membrane to increase cell permeability. From the three, amphotericin B is the one more commonly used and is one of the oldest antifungals available. Until recently, it has been the gold standard for the treatment of IPA, candidiasis, and cryptococcosis. Amphotericin B has a distribution half-life of 0.9 – 1.5 hours and a long elimination half-life, which varies depending on the species and is caused by much of the administered dose depositing in tissues, especially in fat. Intravenous amphotericin B should be used with caution, as it can cause nephrotoxicity and phlebitis. Other possible side effects include anorexia, anemia, cardiac arrhythmias, hepatic dysfunction, and hypersensitivity reactions. Liposomal amphotericin B has been associated with lower nephrotoxicity in human studies, however cost is high. Also, *in vitro*

studies have revealed that some *Aspergillus spp*. and most *Fusarium* and *Scedosporium* spp. isolates are only moderately susceptible or resistant to amphotericin B.

Azoles destroy fungi by inhibition of ergosterol biosynthesis in the fungal cell membrane. The main cellular target of azoles is C14α-demethylase in the ergosterol biosynthetic pathway. The two main azoles used for the treatment of invasive fungal infections are imidazole (miconazole, ketoconazole) and triazoles (fluconazole, itraconazole, enilconazole, voriconazole). Miconazole (topical 2%) has been used in the resolution of 4 cases of guttural pouch mycosis and as part of successful multimodal therapy against nasopharyngeal *Pseudallescheria boydii*[43]. Enilconazole has been used topically in the successful treatment of guttural pouch mycosis and via nebulization for resolution of *Scopulariopsis* spp. pneumonia[35]. Ketoconazole is absorbed poorly in the non-acidified form but can be acidified for better absorption. Itraconazole is absorbed well orally and is effective against yeasts, such as *Histoplasma* spp. and *Blastomyces* spp., and *Aspergillus* spp., with no detectable side effects. The use of compounded itraconazole is not recommended because of its poor stability and highly lipophilic nature. In addition, itraconazole is a weak base and becomes minimally soluble in aqueous solutions only at a low pH[44], which the pH in the stomach of horses may not be sufficiently acidic and may be too variable to ensure consistent absorption of the drug[44,45]. Newer oral formulation of itraconazole in combination with cyclodextrins have improved absorption, however because of the low concentration of drug in the solution, a large volume must be administered, which makes it inconvenient for horse owners[44]. Oral fluconazole yields concentrations in plasma, cerebrospinal fluid, synovial fluid, aqueous humor, and urine more than the MIC reported for several equine fungal pathogens. Fluconazole, however, reportedly has minimal activity against filamentous fungi (*Aspergillus* spp. and

Fusarium spp.). Voriconazole, a new broad-spectrum triazole antifungal agent, was approved for use in human medicine in 2002. It is now considered the drug of choice for initial treatment of invasive aspergillosis, candidiasis, cryptococcosis, and serious fungal infections caused by *Scedosporium apiospermum* and *Fusarium* spp. in patients that are unable to tolerate or are refractory to other therapeutic agents[15,25].

Another common treatment option used in cases of fungal diseases include the use of iodides. Iodides have little direct *in vitro* antibiotic effects, but they seem to have beneficial effect on the granulomatous inflammatory process. Although several successful cases are reported in which iodides were used as primary or adjunctive therapy, overall efficacy is considered limited. Even though treatment is inexpensive, toxicity can occur. Toxicity is characterized by excessive lacrimation, nonproductive cough, increased respiratory secretions, and dermatitis. It is not safe to administer to pregnant mares as it may cause congenital hypothyroidism in foals. Orally administered iodine is available in 2 forms. Inorganic potassium iodide is available only as a chemical grade and is unstable in the presence of light, heat, and excessive humidity. Organic ethylenediamine dihydriodide is commercially available[15].

Prognosis for horses suffering from fungal infections involving the respiratory system depends on a variety of factors that include the causative organism, host immune status, primary disease process, and the stage of the disease at the time of diagnosis, and owner commitment to invest in therapy. In cases where disease is advanced, complicating factors are marked, or therapeutic options are limited, prognosis for disease resolution may be more guarded[10].

1.4 Voriconazole Use in Treatment of Fungal Respiratory Diseases in Humans and Animals

For many years, the standard therapy for IPA in humans was amphotericin b, although responses are suboptimal (reported less than 40%) in severely immunosuppressed patients[46]. As previously mentioned, amphotericin b is associated with multiple side effects. In a way to improve the prognosis of fungal infections and find better antifungals with less side effects, voriconazole was created.

Voriconazole is a second-generation triazole, broad-spectrum antifungal that is structurally related to fluconazole[47]. In comparison to fluconazole, one of the triazole rings was replaced with a fluorinated pyrimidine and an α-methyl group was added, which resulted in expanded activity[48]. The mechanism of action is the inhibition of cytochrome P450 (CYP 450) - dependent 14α-lanosterol demethylation, which is necessary for ergosterol synthesis. This affects the synthesis of the cell membrane of the fungi and leads to cell death. For yeasts, voriconazole appears to be fungistatic, however, for a variety of filamentous organisms, may be fungicidal[48].

This azole is active against a broad spectrum of common pathogenic fungi such as, dermatophytes, opportunistic yeasts (*Candida* spp., *Cryptococcus neoformans*), opportunistic filamentous fungi (*Aspergillus* spp., *Fusarium* spp.) and dimorphic fungi (*Histoplasma, Coccidioides, Blastomyces, Sporothrix*). Isolates with an MIC of ≥ 0.5 μg/ml are considered susceptible. Voriconazole exerts a time-dependent fungicidal activity against *Aspergillus* spp. in vitro[49].

Voriconazole is available in intravenous (IV) and oral formulations. It is metabolized by the liver and, unlike fluconazole and amphotericin B, does not depend on renal function for excretion. However, the IV formulation contains sulfobutyl ether β-cyclodextrin sodium, which is excreted by the kidneys and tends to accumulate in patients with renal failure. Unlike itraconazole, voriconazole is not dependent on gastric acid for absorption and the drug is entirely absorbed in dogs and horses after oral administration[49,50]. Voriconazole has exceptional tissue penetration and distributes widely into body fluids[51].

Generally, voriconazole is well tolerated in humans, and is considered to be superior to amphotericin B for the treatment of patient with IPA, as well as safer than amphotericin B in patients with renal dysfunction[52]. Transient visual disturbance is the most common adverse effect occurring in 20-40% of human patients. The effect is dose related and it is seldom necessary to stop therapy. Drug interactions are common, which is also an issue with other triazoles. There have been reports of cats treated with voriconazole that develop neurologic signs, such as ataxia, and in some cases, progressed to paraplegia[53]. These cats also developed visual abnormalities including mydriasis, decreased to absent pupillary light responses, and decreased menace response. Arrhythmias and hypokalemia were also noted[53]. In horses, urticaria has been the only side effect reported with the use of voriconazole[51].

In humans, voriconazole is approved for the treatment of IPA and serious infections caused by *Scedosporium* spp., *Fusarium* spp., or invasive fluconazole-resistant *Candida* spp. Pulmonary infections caused by *Aspergillus* species are associated with significant morbidity and mortality in immunocompromised patients. Voriconazole has been reported to have a good outcome when

treating patients with cystic fibrosis that developed secondarily allergic bronchopulmonary aspergillosis, or in cases of people with allergic bronchopulmonary aspergillosis and allergic fungal sinusitis, also caused by aspergillosis[54], all these with oral therapy.

In horses, several pharmacokinetic studies of voriconazole have shown that the drug has an excellent absorption after oral administration and a long half-life[52], as well as a quick and wide distribution in the body and body fluids[50,51]. Recently, this antifungal drug has become a more popular therapeutic option for many fungal infections in horses and other domestic animals.

Due to the concern of side effects caused by long-term therapy, which is commonly required in cases of fungal pneumonia, aerosolized delivery of voriconazole has been investigated, especially for treatment and/or prevention of IPA. The purpose is to achieve a high local drug concentration with minimal systemic exposure[41]. Inhaled voriconazole reduces histological manifestations of IPA in rodents[38] and it has been proposed that a favorable lung tissue to plasma concentration ration is obtained through this route of administration[55]. Case reports demonstrating a positive outcome with the use of inhaled voriconazole have been reported in people with different respiratory fungal infections[39,56]. In horses, there are only case reports describing the use of systemic voriconazole for pulmonary aspergillosis[34], but experience with the use of aerosolized voriconazole has yet to be described.

1.5 Considerations for Distribution of Drugs to the Respiratory System

In order to obtain the best outcome when treating mycotic respiratory infections is important not only to know the drugs available, but also understand how these drugs get distributed

in the respiratory system. Infections of the respiratory system can be difficult to treat with systemic therapy due to anatomical barriers that prevent the drug to reach the appropriate concentration in the airways or lungs. Also, the drug may be metabolized or excreted prior to having any effect in the lungs. Studies in animals and humans have shown that serum antimicrobial concentrations are not good indicators of drug concentrations in the lung. Drug concentrations obtained from the pulmonary epithelial lining fluid (PELF) may not reflect concentrations achieved in the parenchyma or even the bronchial epithelium. Nonetheless, the PELF concentration is the best indicator of a drug's penetration into the respiratory system. Drug-specific factors, including protein binding and tissue penetration, also affect the disposition of drugs to the lungs[57].

Drug penetration is also affected by the disease state itself. When there is inflammation, this causes vasodilation that enhances drug delivery, however, it may also increase the thickness of the alveolar epithelium which will impair drug delivery to the diseased area. In addition, high drug lipophilicity, low protein binding and a relatively basic ionization of the drug enhance the penetration of drugs to the lung as these characteristics improve passive diffusion through any membrane, and especially in the face of the tight junctions between cells that predominate in the lung[57,58].

It is important to determine the means by which the horse has acquired a respiratory infection as it has important implications for treatment. Making this distinction aids to determine if the infection is systemic, thus it is necessary to consider drug distribution to the rest of the body, or if the infection is localized to the lung, hence the lung is the primary target. Distribution of systemically administered drugs is actually greater to the airways than to the alveolar space, as the

alveolar epithelium is well supplied with zonula occludens that prevent many substances from crossing the alveolar barrier[57].

Due to these factors affecting the distribution of drugs to the respiratory system, administering antimicrobials by the aerosolized route is an attractive therapeutic option. Nonetheless, there are important factors that may inhibit aerosol delivery to the lung, such as atelectasis, bronchoconstriction, excessive mucus production, and inflammation. The physical characteristics of the medication may also affect the ability of the aerosol to travel within the airways, and include particle size, viscosity, surface tension, osmolality, tonicity and pH[41,59]. The particle size or mean mass aerodynamic diameter (MMAD) is one of the most critical characteristics of aerosol therapy. Large aerosols (> 10 µm) are trapped by the ciliated epithelium of the URT and sneezed or swallowed, or deposited in larger airways in association with turbulent airflow, and do not effectively reach the lower airways. An MMAD of 1-5 µm is the ideal size that allows for drug deposition into the small airways and alveoli[60].

Knowing the characteristics of the medication and the different devices available for aerosol delivery is needed to ensure appropriate distribution of the drug to the respiratory system when using inhalation therapy.

1.6 Inhalation Therapy in Horses

Inhalation therapy allows a rapid deposition of a high concentration of medication as an aerosol in the peripheral airways and avoids or minimizes systemic side effects. In equines, it has also been implemented to optimize treatment efficacy and reduce the dosage of drugs required[61].

However, delivery of drug by the aerosol route requires that the airways are patent and sometimes, depending on the localization of the infection within the respiratory system, systemic administration of antimicrobials will also be required as it may be that the combination of both therapies will produce a synergistic effect[57].

An aerosol is a suspension of solid or liquid particles dispersed in gas. The onset of action of aerosolized drugs is relatively rapid; however, the effects are usually short-lived. This is because the aerosolized drugs are partially degraded in the lung, cleared from the respiratory tract by the mucociliary escalator, and absorbed into the blood stream where they are disposed of by breakdown and excretion as are systemic drugs[59].

Inhaled particles can be deposited by inertial impaction, gravitational sedimentation or diffusion depending on their size. Particles ranging from 1 to 5 μm suspended in gas, referred to as therapeutic aerosol, maximize deposition in lower airways deeper in the lung[59]; in addition, a maximal distal deposition occurs when the patient takes slow, deep breaths, with large tidal volumes. Deposition occurs throughout the airways, but inertial impaction usually occurs in the upper airways or bigger bronchi[62] and when inhaled large size particles (> 10 μm)[59]. Deposition by gravitational sedimentation occurs with midsized and small particles, and predominates in the smaller bronchi and bronchioles where air velocity is low[59,63]. In the alveolar region, air velocity is minimal, and thus the contribution to deposition by inertial impaction is null. Particles in this region have a longer residence time and are deposited by both sedimentation (if MMAD > 0.5 μm) and diffusion (if MMAD < 0.5 μm). Particles that are not deposited during inhalation are exhaled.

Side effects of inhalation therapy are related to the physical and chemical characteristics of the aerosol or the formulation of the solution as it can affect the characteristics of the aerosol produced[64]. The inspired particles must be physiologically compatible with the lung membranes, thus be isotonic, have a neutral pH, and be non-immunogenic to avoid airway hyper-responsiveness, cough or bronchoconstriction, or inflammation[55,61,65].

The available forms of drug delivery by the inhalation route for horses include vaporizers, nebulizers and pressurized cartridge dispensers for administration of aqueous or alcoholic solutions as well as powders in the form of aerosol. Nebulization is used for the delivery of medications formulated as a liquid, either as a solution or suspension. The different types of nebulizers include jet, ultrasonic and mesh[59].

In horses, aerosolized therapy has been used for many years, principally in cases of bacterial pneumonia and lower airways inflammatory disease/asthma to deliver antibiotics, anti-inflammatories, or bronchodilators[57,59,66].

1.6.1 Inhalation versus Systemic Route of Drug Administration

Inhalation therapy has started to play a more important role in the management of respiratory disorders. There are a few respiratory pathologies that could be treated or prevented avoiding systemic drug administration, for example the use of inhaled voriconazole for prevention of IPA in humans that are at high risk of developing the disease[38] or with the use of inhaled corticosteroids for treatment of equine asthma[67]. As previously mentioned, the blood-bronchial barrier limits the access of systemically administered drugs to the airway lumen and to the cells

lining the lower respiratory tract. Thus, in order for most medications to achieve drug penetration into the lungs, a high systemic dose is often required[59].

Some drugs may result in undesirable and sometimes life-threatening side effects, such as the case when bronchodilators (parasympatholytic or sympathomimetic drugs) are used in cases of asthma and can cause tremors, tachycardia, sweating, decreased gastrointestinal motility, and cardiac arrhythmias, when used in large systemic doses. Administration of these drugs through the inhalational route minimized the risks of these side effects[59]. Drugs administered by aerosol obviate absorption, bypass degradation in the gastrointestinal tract and liver, avoid detrimental effects on the gut flora, and allow the use of drugs that are not bioavailable when administered orally[59].

Although drugs administered through the inhalational route can still cause adverse reactions, such as anaphylaxis, hypersensitivity, idiosyncratic reactions, overdose, cumulative effects, and toxicity, they are less likely to do so than the same drugs administered systemically. Tolerance, defined as resistance to standard dosages of drugs, can also occur with inhalational therapy. Tolerance of inhaled drugs may be manifested as tachyphylaxis, which is when the biological response to a drug diminishes rapidly, sometimes even with the initial dose and may be due to receptor phosphorylation; as resistance, which indicates that there is loss of effectiveness of an antimicrobial drug; or paradoxic effect, that is when the effect of a chemical substance is the opposite to the effect that would normally be expected; or rebound phenomenon that is when the symptoms that were absent or controlled while taking the medication, re-emerge when the medication is discontinued or reduced in dosage[59].

In cases where long-term therapy is required, aerosol treatment is often a more acceptable means by which owners can manage their horses at home, for example in horses requiring bronchodilators or steroids for weeks when being treated for asthma. Treatment of infections of the lower respiratory tract with aerosolized antimicrobial drugs may avoid antibiotic-induced colitis associated with systemic administration, or other side effects such as nephrotoxicity with aminoglycosides, and can also enhance the efficacy of certain antimicrobials and increase their concentration in the diseased area[59].

One of the disadvantages of inhaled therapy is the delivery of a precise dose of a medication. Because the drug deposition may variate depending on the medication used, the determination of half-life of inhaled drugs is difficult. The efficacy of drugs administered through inhalation is best assessed by the observation of a desirable drug effect. Knowledge of the half-life is helpful, because for an adequate response, many drugs are administered at intervals that are approximately twice that of the half-life. Drugs administered through inhalation tend to have a shorter duration of effect than when administered through the systemic route. Thus, more frequent administration may be necessary to attain a similar desired effect. Drug deposition of the inhaled drug may be even more unpredictable in diseased lung. Proper drug distribution is affected by abnormal breathing patterns, bronchoconstriction, airway secretions, and coughing. Therefore, control of coughing and bronchospasm is important to obtain an effective distribution of any drug through the inhalation route[59].

1.6.2 Delivery of Aerosols

In order to choose inhalation as the route of drug administration one should know which are the different devices available, how they work, and the formulations of drug available to attain the best outcome. As stated earlier, there is an important relationship between particle size and deposition of the drug in the lower airways, which can significantly variate depending on the device used[68]. Therapeutic aerosols are produced using different types of inhalation devices. Some of these devices available for horses include vaporizers, nebulizers and pressurized cartridge dispensers.

Vaporizers are the oldest form of inhalation therapy and are currently used primarily for the purpose of humidification of airway secretions as they are thought to increase the fluidity of airways secretions making it easier to eliminate thick mucus by the ciliary escalator and coughing[59]. They deliver the drug vaporized in steam and their main disadvantage is that they produce mainly large particles (10-50 μm) that to a large extent settle on the nasal mucosa by gravitational settling and inertial impaction, and consequently making the drug deposition highly variable. Because of this, they have been replaced by other devices for medication delivery[69].

Nebulizers generate aerosols by different mechanisms, depending on the type. There are three types of nebulizers: jet, ultrasonic, and mesh nebulizers.

- Jet nebulizers use a powerful dry air compressor to produce a spray from breaking up the liquid into small particles[66]. If large droplets are formed, they return to the reservoir and enter the next cycle, whereas smaller particles are carried in inhaled air. The advantage of this type of nebulizers is their lower cost, but they are loud, provide a slow delivery rate, the particle size is

highly variable, and relatively large volumes of liquid are required for a small amount of actual drug delivery. In humans, the reported deposition of drug by jet nebulizers is approximately 7% to 7.5% of nebulized volume, which is similar to slightly lower in horses[70].

- More common devices used in horses are ultrasonic and mesh nebulizers. Ultrasonic nebulizers use piezoelectric crystal vibrations to nebulize a pool of liquid into a cloud of mist. The particle size is determined by the frequency of the vibration, so higher frequencies create smaller droplets, but droplet size and deposition are determined by several factors, including individual drug characteristics, nebulizer specifications, and tubing length and diameter. The advantages of this type of nebulizer are the faster delivery and creation of more specific droplet size than jet nebulizers. However, they are more expensive than jet nebulizers, and they generate heat, which may degrade the medication. In humans, the reported deposition of drug is around 5% of nebulized volume, which is similar to what is reported in equines[64,70].

- Lastly and very similar to the previous device, mesh nebulizers have the same piezoelectric element but with an added mesh, that are in contact with each other. The piezoelectric element creates a rapid physical vibration of the mesh (which was a minimum of a 1,000 small holes within it) that force the liquid through these holes creating the desired small particle mist. In comparison to ultrasonic nebulizers, mesh nebulizers have a self-contained power source, but otherwise they have similar advantages. Both jet and ultrasonic nebulizers require good sanitation, as if this is not performed, may result in deposition of bacteria into lower airways.

Pressurized metered dose inhalers (pMDIs) are used for administration of aqueous or alcoholic solutions, as well as powders in the form of aerosol. They provide a method of ensuring administration of an accurate amount of medication by delivering a set amount of drug per actuation or puff. Although this method is thought to be precise, some variability of delivery may still occur. For example when the user fails to adequately shake the pMDI before using it or when actuation of the pMDI is not coordinated with inhalation. That is why horses require the use of a delivery device (a spacer) for this method. The pMDI is actuated into the spacer with a one-way inspiratory valve and the drug is inhaled through the spacer to facilitate administration of the aerosol and to increase small dimeter particles inhalation[66]. In humans, incorrect coordination results in oropharyngeal deposition of the drug rather than pulmonary deposition, but correct coordination results in 60% of the drug reaching the lungs. The type of propellant and the form of delivery influence the relative deposition of the drug in the lung. The type of propellant influences the relative deposition of drug in the lung. Currently, chlorofluorocarbon propellant is no longer available, and only the hydrofluoroalkane propellant is available in the United States[59].

1.6.3 Pharmacokinetics of Aerosolized Drugs

For a better understanding of the use and efficacy of inhalation therapy, it is imperative to understand what happens with the drug when it reaches the lower airways and lungs. An inhaled drug may be eliminated from the lung by mucociliary or cough clearance to the gastrointestinal tract, by passive or active absorption into the capillary blood network, or by metabolism in the mucus or lung tissue[71]. These mechanisms have an important role in determining the drug's duration of action in the lungs and the airway selectivity of the inhaled drug. Inhalation therapy tends to require multiple dosing as their effects tend to be short-lasting.

Once the drug aerosol has been deposited onto the lung surface, the immediate fate of the drug depends on its physical state. Drugs in aqueous form are readily absorbed by the airway epithelial lining, compared to drugs in a suspension form that are not easily absorbed and are eliminated faster via the mucociliary escalator or cough clearance[71]. Most drugs administered through the inhalation route into the lung are relatively bioavailable. Any delay in absorption may result in mucociliary clearance of the drug before it has a chance to achieve full effect; the more soluble the drug, the faster it is absorbed. Also, slowly dissolving drug deposited in the alveolar region may be phagocytosed by alveolar macrophages, which expel particles either by transporting them along the alveolar surface to the mucociliary escalator, or by translocation to tracheobronchial lymph or by internal enzymatic degradation[62,72]. Phagocytosis is optimal for particles of 1.5-3 μm in size[73].

The lungs have some ability of drug clearance, however they are not as efficient as the liver. Metabolism in lungs differs significantly from the intestinal-hepatic metabolism. The expression levels of enzymes are generally lower and the expression patterns of drug-metabolizing enzymes differ. The most abundant liver cytochrome P450 (CYP) enzyme, CYP3A5, is expressed to a lower degree in pulmonary tissue, where the isoform CYP3A5 is considered more important[74].

The routes of drug absorption across the epithelium include passive and active transport mechanisms involving paracellular and transcellular, pore formation, vesicular transport, and drainage into the lymphatics, depending on the drug and site of absorption. The absorption of the drug into the systemic circulation is of concern with drugs that have undesirable side effects, such

as immunosuppression from corticosteroids or the anticholinergic effects of atropine-like drugs. Even though drugs administered into the airway can be systemically absorbed through alveolar capillaries and into the bloodstream, the zona occludens that limits entry of systemically administered drugs into the airways generally allows little of the inhaled drug to reach the circulation.

It is important to consider that the use and effectiveness of aerosolized therapy may be altered by physiologic changes in the lungs, such as age-related changes or pathologic conditions as a result of the disease process, and therefore, the amount of drug that can be deposited in the lung may be decreased.

1.7 Justification of the Study

Fungal respiratory diseases are considered uncommon in horses, however this can vary depending on the geographic localization, and when present, they can be life threatening. The diagnosis and treatment of fungal respiratory infections are challenging for equine veterinarians, and the prognosis for complete resolution of infection is often guarded[15].

One of the most common organism isolated in equine respiratory fungal disease such as fungal pneumonia, is *Aspergillus* spp. Similarly in humans, *Aspergillus* spp. cause a well described fungal disease called invasive pulmonary aspergillosis, which is associated with high mortality rates in immunosuppressed human patients despite treatment. Efficacy of treatment protocols for fungal respiratory infections in horses have not been thoroughly studied[37]. Therapeutic options to treat horses with fungal diseases are few due to the cost of the drugs, the systemic side effects, and

the drug disposition. As an example, amphotericin B is a commonly, well-known antifungal medication that is effective against *Aspergillus* spp. and many other common pathogenic fungi, but can have several adverse effects in horses such as nephrotoxicity, anorexia, anemia, cardiac arrhythmias, hepatic dysfunction, hypersensitivity reactions, and phlebitis[15,51].

In the search of newer and more efficient antifungal drugs, voriconazole was produced. Voriconazole is a broad-spectrum antifungal agent used against a variety of clinically important yeasts and molds (*Aspergillus* spp., *Candida* spp.), and is considered the first line treatment drug for IPA in humans. Voriconazole is usually well tolerated in humans, though there are dose-related side effects reported like transient visual disturbance, urticaria, hepatotoxicity and neuropsychiatric symptoms[75]. More recently in horses, several studies have looked at the use of voriconazole as another therapeutic option for systemic fungal disease, and the drug has shown very good bioavailability when administered orally with wide distribution in body fluids[50-52]. There is a study performed in 6 mares that received oral voriconazole once daily for 14 days, and only 1 horse showed an adverse reaction on day 2 of medication, which was urticaria and that was treated and resolved, but further monitoring in sick horses needs to be done.

Mycotic infections tend to require a long-term therapy which is usually expensive and may have significant adverse effects in the patient. In recent years, researchers in human medicine have focused attention on inhaled delivery of medications for the treatment of respiratory fungal infections, as administration via nebulization ensures local disposition of the drug in the affected areas, reducing dose requirements, while potentially limiting systemic adverse effects[59]. Targeted

drug delivery of aqueous voriconazole solutions has been proven to produce high tissue and plasma drug concentrations as well as improved survival in a murine study of IPA[76].

In equine medicine, inhalation therapy has been used by practitioners for several years, mainly to deliver antibiotics, anti-inflammatory and bronchodilators[59,61,69]. Because ease of administration with new, commercial nebulizers, many practitioners have implemented this form of therapy to deliver systemic drugs directly to the lungs without research evidence and therefore not knowing if it is really effective. Based on current literature, this would be the first study in horses performed to measure the concentration of an antifungal drug delivered via nebulization. This proposal has potential to positively impact the health of horses worldwide and has important implications for practitioners, by developing a feasible therapy with minimal adverse effects, which may improve the prognosis for respiratory fungal diseases.

Chapter 2. Hypotheses and Objectives

Specific Aim 1: Clinical feasibility of nebulized voriconazole and drug deposition in the lower airways

A. Hypothesis Aim 1:
    a. Concentrations of voriconazole after delivery by nebulization would reach clinically significant concentrations in the PELF versus the plasma.
    b. Nebulization of voriconazole solution would be well tolerated and would not cause inflammation of the lower airways.

B. Objectives Aim 1:
    a. Determine voriconazole concentrations in plasma and PELF after delivery by nebulization.
    b. Determine if voriconazole concentration in the PELF and plasma would reach the MIC for *Aspergillus* spp. ($\geq$ 0.5 μg/ml) after nebulization.
    c. Determine if nebulization of Vfend® solution induced inflammation of lower airways in adult horses by evaluation of BALF cytology.

Specific Aim 2: Voriconazole deposition in the upper airways after delivery by nebulization

A. Hypothesis Aim 2:
    a. Concentrations of voriconazole in nasopharyngeal wash samples after delivery by nebulization would reach clinically significant concentrations compared to plasma, but similar to PELF.

B. Objective Aim 2:

a. Determine the concentration of voriconazole in NPW samples after delivery by nebulization and determine if [VRC] concentrations would reach the MIC for *Aspergillus* spp. (≥ 0.5 μg/ml) after nebulization.

Chapter 3. Clinical Feasibility and Airway Deposition of Nebulized Voriconazole in Healthy Horses

3.1 Introduction

Fungal pneumonia or pneumomycosis, is an unusual, yet potentially life-threatening condition in horses. Fungal infections of the upper respiratory tract [2] are more common than in the lower respiratory tract (LRT) and may affect the nasal passages, paranasal sinuses, and guttural pouches[15]. These infections are acquired most frequently via inhalation of the causative organism; however, some cases are thought to arise by penetration of fungi through an inflamed gastrointestinal tract or open wound with subsequent hematogenous spread to the lungs[13]. In most cases, pneumomycosis develops secondary to the use of immunosuppressive drugs for treatment of diseases such as inflammatory bowel disease and equine asthma[13,15].

In horses, one of the most common organisms isolated in fungal respiratory diseases are species of the genus *Aspergillus*, which are ubiquitous in the environment[13]. Invasive pulmonary aspergillosis (IPA) is a fungal disease of the lungs associated with high mortality rates in immunosuppressed horses[20,37] and in people[38,77], despite treatment. Diagnosis and treatment of fungal respiratory infections are challenging for equine veterinarians, and the prognosis for complete resolution of infection is often guarded[15]. Treatment requires long-term systemic administration of antifungal medication with potential severe side effects, and efficacy of treatment protocols for pneumomycosis in horses has not been thoroughly studied[37].

Voriconazole (VRC) is a broad-spectrum triazole antifungal agent used against a variety of clinically important yeasts and molds (*Aspergillus* spp., *Candida* spp.), and is considered the first line treatment drug for IPA in people[78]. Voriconazole is usually well tolerated in humans, although there are dose and duration related side effects such as transient visual disturbance, urticaria, hepatotoxicity, and neuropsychiatric symptoms[48,75]. In horses, side effects associated with VRC administration have not been reported, but this may be due to a lack of information on long term (more than 14 days) systemic administration[51]. Rather than systemic administration, the most common use of VRC by equine practitioners in horses is the aqueous solution (Vfend®) for topical or intralesional treatment of ocular infections.

In recent years, researchers in human medicine have focused attention on inhaled delivery of medications for the treatment of respiratory fungal infections, as administration via nebulization ensures local disposition of the drug in the affected areas, and reduces systemic dose requirements and the potential for systemic adverse effects[41]. Targeted drug delivery of VFend® demonstrated high tissue and plasma drug concentrations, as well as improved survival in a murine study of IPA [38,55], and detectable tissue and plasma drug concentrations in a human study[75]. Based on current literature, there are no studies in horses measuring the concentration of an antifungal drug delivered via nebulization. Recent developments in nebulizer technologies for use in horses have resulted in the development of mesh nebulizers, such as the Flexineb®. This type of nebulizer has the advantage of being silent and battery powered making it easy to use and portable. The medication cup is comprised of a gold-plated metallic mesh containing very small holes that when energized produces high frequency vibrations which generate a fine aerosol mist. The particle size distribution within the aerosol is largely determined by the size of the holes within the vibrating

mesh[79]. This device is commonly used in equine practice and has successfully been used in several equine studies[2,80] to deliver medication into the lower airways.

The primary objective of this study was to determine feasibility of delivery of a common, commercially available aqueous VRC solution (Vfend®) to horses by nebulization using a common, commercially available equine nebulizer (Flexineb®), and to determine concentrations of VRC in lower airway samples and plasma. The hypothesis was that VRC delivery by nebulization would be well-tolerated and would produce clinically significant concentrations (above the MIC for *Aspergillus* spp. 0.5 µg/mL) of VRC in the lower airways that were higher than plasma concentrations. In case of rejection of the hypothesis due to low VRC concentrations in the lower airways, a secondary objective was to measure VRC concentrations in the upper airway through collection of nasopharyngeal wash fluid (NPW) samples.

3.2 Material and Methods

3.2.1 Horses

The study was approved by the Institutional Animal Care and Use Committee (PRN 2018-3279). University-owned, adult mixed breed geldings were included in the study. Mean body weight was 552 kg (range: 461 - 639 kg). Mean age of the horses was 10 years (range: 6 – 12 years). The horses were determined to be healthy for inclusion in the study based on normal physical examination (including re-breathing examination) (**Figure 1**), complete blood count (CBC), serum biochemical analysis (including bile acids concentration), and bronchoalveolar fluid (BALF) cytological examination. This data (BALF analysis) was used as baseline data for each individual and was obtained within 2 weeks prior to drug administration. Criteria considered for

exclusion included abnormal findings in the physical examination, signs of inflammation or infection on the BALF analysis or bloodwork, or the receipt of any medication within 30 days of enrollment.

Horses were maintained on pasture and acclimated to an individual stall in the barn 24 hours prior to the study. Water and bermudagrass hay were available *ad libitum*. Physical examinations were performed twice daily throughout the study. Horses were returned to their regular pasture the day after the study was performed. Each horse stayed in a stall for a total of 3 days.

Figure 1: Re-breathing examination performed in a horse as part of the initial physical examination required for inclusion in the study.

3.2.2 Voriconazole

3.2.2.1 Aerosol Particle Size

The particle size of the aerosol created by the equine nebulizer (Flexineb E2®; JS Equine Division, Union City, TN, USA) was measured by the manufacturer using a spray particle and spray droplet size analyzer (Spraytec®; Malvern Panalytical Ltd., Enigma Business Park, Grovewood Road, Malvern, UK). Because there is usually ± 5% deviation from one measurement to another (in terms of % of particles), the analyzer takes measurements every 3 seconds and runs the test for a minute, thus getting 20 measurements per minute, and an average measurement is obtained at the end. To determine the flow rate obtained when nebulizing Vfend® solution, saline was used as a baseline for comparison, and two concentrations (10 mg/mL and 6.25 mg/mL) of Vfend® solution were tested. Based on those results, the 6.25 mg/ml concentration was used for all nebulizations.

3.2.2.2 Preparation of VRC and Delivery by Nebulization

Voriconazole lyophilized sterile powder for injection 200 mg (Vfend®; Pfizer Inc., New York, NY, USA) was reconstituted using 32 mL of sterile water for injection to make a final concentration of 6.25 mg/mL[55]. Voriconazole was delivered using a portable mesh nebulizer (Flexineb E2®; JS Equine Division, Union City). A standard (grey, Standard Flow Medication) cup and fast (green, Fast Flow Medication) cup (commercially available) designed to increase the speed of nebulization, were provided by the company for this study (**Figure 2**). The fast cup was designed to use with difficult to nebulize, high viscosity medications in order to maximize deposition, reduce condensation in the mask (decrease waste), and reduce the delivery time.

Figure 2: A horse from the study during nebulization using the Flexineb mask.

3.2.3 Samples

3.2.3.1 Plasma

Plasma samples for measurement of VRC and urea were collected through a 14-gauge, 2-inch intravenous (IV) jugular catheter (Surflo®; Terumo Medical Corp., Somerset, NJ, USA), which was placed aseptically in the horses on the day of drug administration. Blood samples were collected in sodium heparin tubes and kept on ice until centrifugation within 1 hour of collection. These samples were spun at 2106 relative centrifugal force (rcf) for 10 minutes. The resultant plasma was stored in duplicate at -80 °C until batch analysis.

3.2.3.2 Bronchoalveolar Lavage

Horses were sedated with detomidine hydrochloride (Dormosedan®; Zoetis, Parsippany, NJ, USA; 0.01 mg/kg IV) and butorphanol tartrate (Torbugesic®; Zoetis, Parsippany, NJ, USA; 0.01 mg/kg IV) and a bronchoalveolar lavage catheter (BAL 240: 10 mm OD, 2.5 mm ID, 240 cm; Mila International Inc., Florence, KY, USA) was passed through the nasopharynx, lodged blindly in a bronchus, and the cuff was inflated (**Figure 3**). Using syringes, 60 mL of 0.5 % lidocaine were instilled, followed by 250 mL of sterile 0.9% sodium chloride solution (saline). The fluid was retrieved immediately by syringe aspiration and placed on ice until processing within 1 hour of collection. The BALF was pooled in a sterile graduated cylinder, and the amount of fluid recovered was recorded. A portion of the BALF sample was placed into an EDTA tube for cytological examination by a board certified clinical pathologist, and 6 mL of BALF was frozen at -80 °C for batch evaluation of antifungal activity via the agar disk diffusion method[81]. The remainder of the BALF was centrifuged at 2106 rcf for 10 minutes to separate the supernatant and cell pellet for [VRC] analysis. Once samples were obtained, the supernatant was frozen as triplicate at -80 °C and the cell pellet was re-suspended in 1 mL of phosphate buffered saline (PBS) to be frozen as triplicate at -80 °C. For BALF analysis, cytospins were prepared, and 400 cells were counted. The percentages of inflammatory cells (macrophages, small lymphocytes, neutrophils, eosinophils, and mast cells) were recorded[82,83].

3.2.3.3 Nasopharyngeal Wash

The nasopharyngeal wash was performed using a 14-French X 16-inch red rubber catheter (Rusch®; Teleflex, Morrisvile, NC, USA) and 60 mL of sterile 0.9% saline. The catheter was introduced through one of the nostrils to the nasopharynx and 60 mL of sterile saline was infused.

A sterile cup was used to collect the fluid flowing from the nostrils. This fluid was kept on ice until stored (within 1 hour of collection) in triplicate at -80 °C until batch analysis.

Figure 3: Bronchoalveolar lavage procedure to obtain fluid from the alveoli in a sedated horse.

3.2.4 High-Performance Liquid Chromatography (HPLC) Assay

Plasma, BALF supernatant and cell pellet, and NPW samples were analyzed by reverse HPLC as previously described[51]. Briefly, the HPLC system (Waters – Alliance HPLC; Waters Corporation, MA, USA) consisted of a separation module (Waters e2695), photodiode array detector (Waters 2998), column (Thermo BetaBasic-18, 4.6 mm x 15 cm, 5 µm; Bellefonte, PA, USA), silica guard column (4 mm x 1.25 cm), and computer for data collection and analysis (Gateway 2000, P4D-66, Irvine, CA, USA). The mobile phase consisted of 35% 0.1 M N, N, N', N'-tetramethylenediamine (Fisher Scientific, Inc., Waltham, MA, USA) and methanol (Fisher

Scientific, Inc.), at a flow rate of 1.0 mL/min at room temperature. The injection volume was 100 µL. Voriconazole and ketoconazole (the internal standard), were detected at a wavelength of 254 nm, and the retention times were 3.7 and 13.5 minutes, respectively. Calibration standards with voriconazole concentrations in plasma, cell pellets and supernatant from BALF, and NPW samples ranging from 0.02 to 10 µg were prepared. Plasma, BALF, and NPW samples collected at baseline prior to administration of voriconazole were used as the blank controls. A dilution correction using urea was applied to any BALF supernatant samples with detectable VRC concentrations in order to estimate pulmonary epithelial lining fluid (PELF) concentrations according to previously published methods[51]; this correction was not applied to NPW samples as there are no published assessments using this technique in that fluid in horses.

3.2.5 Extraction

To 500 µL of each sample, 15 µL of internal standard solution was pipetted and vortexed for 10 minutes. Voriconazole and internal standard were extracted with 4 mL ethyl acetate, as previously described[[51]. The dried residue was reconstituted with the mobile phase and transferred to an HPLC vial and inserted for analysis. Recoveries from plasma and fluids were >100 ± 6% for drug and internal standard. Calibration standards with voriconazole concentrations in plasma, cell pellets and supernatant from BALF, and NPW samples ranging from 0.02 to 100 µg/mL (g) were prepared. Calibration curves of peak area ratios (voriconazole/internal standard) vs. concentration appeared linear with linear regression analysis yielding R^2 values > 0.99. The lower limit of detection for voriconazole was 0.02 µg/mL, and the lower limit of quantification was 0.05 µg/mL. Concentrations below 0.05 µg/mL were reported as below detected. Based on results of at least 5 standard curves [0.05 to 100 µg/mL (g)] and duplicates, the intra- and inter-day variations

were 1.96% and 5.01%, respectively. There appeared no evidence of drug sample carryover in the HPLC methods based on inclusion of blank samples.

3.2.6 Antifungal Activity of VRC from BALF and NPW

Antifungal activity of VRC within the BALF and NPW samples was evaluated via the agar disk diffusion method, as described previously[84]. Briefly, *Aspergillus flavus* (ATCC 204304) was grown on potato dextrose agar slants at 25 °C with an aliquot sub-cultured onto Sabouraud dextrose agar (SDA) and passaged to ensure purity and viability. After four days of incubation, the slants were washed with sterile saline, Tween 20 (Sigma-Aldrich Corp.) was added to improve conidia dispersion and the suspension was adjusted to No. 1 McFarland standard using sterile saline as the diluent. For the inoculum, the cell density of the suspension was adjusted until absorbance reading with a spectrophotometer (Genesys 10S UV-VIS spectrophotometer; Thermo Fisher Scientific, Waltham, MA, USA) was OD530 nm, which equated to 0.4 x 10^6 CFU/mL to 5 x 10^6 CFU/mL. Dilutions were then plated on SDA and incubated at 25 °C for 48 hours to confirm density. The inoculum was swabbed onto Mueller Hinton agar (MHA). Sterile paper disks were impregnated with 100 µL of the unseparated BALF and NPW samples and were placed in triplicate on MHA plates with a voriconazole control disk (1 µg, BD Sensi-Disk; Becton Dickenson and Co., Franklin Lakes, NJ, USA). Plates were incubated at 35 °C and examined at 24 and 48 hours. The diameter of the zone of inhibition around each disk was measured in millimeters. A diameter equal or greater than 17 mm was considered the epidemiologic cutoff value for detection of molds that were susceptible[81,85].

3.2.7 Study Protocol

3.2.7.1 Objective 1: Clinical feasibility of VRC nebulization and VRC concentrations in BALF and plasma

Clinical feasibility was assessed in 6 horses by examining the dose of VRC required to attain detectable concentrations in BALF and PELF, duration of nebulization, tolerance of nebulization and cytological abnormalities in BALF after nebulization. The duration of nebulization and residual fluid volume accumulated in the reservoir at the end of nebulization were recorded for all horses. A fast flow cup was used for delivery of VRC in all horses, except the first horse for whom the standard flow cup was used. Information regarding VRC dose, administration, and sample collection time points for each horse is summarized in **Table 1**. 200 mg of VRC (32 mL at 6.25 mg/mL) was delivered by nebulization to 3 horses (horse 1, 5, 6). This dose was selected based on studies performed in humans using a 40 mg dose[55,75]. Additional VRC doses evaluated for plasma and BALF cell pellet and supernatant VRC concentrations included 500 mg (85 mL at 6.25 mg/mL, horse 2) and 100 mg (16 mL at 6.25 mg/mL, horse 3 and 4). All horses were monitored for coughing, head tossing or shaking, and pawing during nebulization.

Whole blood was collected for VRC plasma concentration analysis immediately prior to nebulization (time 0) in all horses, immediately following nebulization (n = 6), and at 1 hour (n = 5), 2 hours (n = 1), 4 hours (n=1), 6 hours (n=5) and 8 hours (n = 1) post-nebulization. Samples of BALF were obtained immediately following nebulization (n=5), and at 1 hour (n = 1), 6 hours (n = 1) and 8 hours (n = 1) post nebulization. All BALF samples were processed for evaluation of supernatant and cell pellet VRC concentrations and were also analyzed for anti-fungal activity using the agar disk diffusion method. For each horse, cytological examination was performed on

the first BALF sample collected after nebulization and compared to samples collected within 2 weeks prior to drug delivery at the time of screening.

3.2.7.2 Objective 2: Concentrations of VRC in NPW Samples

Information regarding VRC dose and sample collection time points is summarized in **Table 2**. From the horses used in objective 1, an NPW sample was collected at the end of nebulization from horses 2-6, and at 1 and 6 hours post nebulization in horses 3-6. NPW samples were analyzed for VRC concentrations by HPLC.

An additional 5 horses were administered 200 mg (32 mL at 6.25 mg/mL) of VRC solution using the fast cup. Plasma and NPW samples were obtained immediately prior to nebulization, at the end of nebulization, and at 1, 6, and 8 (NPW only) hours after nebulization**.** Plasma and NPW samples were analyzed for VRC concentrations by HPLC, and NPW samples for anti-fungal activity using the agar disk diffusion method.

3.2.8 Data Analysis

Descriptive statistics were calculated where appropriate for measurements of VRC concentration in plasma, BALF cell pellet, BALF supernatant, and NPW. Continuous data were expressed as mean ± SD. For antifungal activity assessment, a positive inhibition was determined if the diameter of the zone of inhibition around each disk was equal to or greater than 17 mm. Data were evaluated for normality using the Shapiro-Wilk test. The percentages of neutrophils, lymphocytes, and macrophages in BALF samples were compared between samples collected before and after nebulization of VRC using a Wilcoxin matched pairs signed rank test.

Concentrations of VRC in NPW samples from 5 horses after nebulization of 200 mg of VRC were compared across timepoints using a Friedman's test with Dunn's multiple comparison test. $P < 0.05$ was considered significant. Data were analyzed with a commercial software program (GraphPad Prism v. 6, La Jolla, CA, USA).

3.3 Results

3.3.1 Particle Size

The particle size for voriconazole using the standard cup was not determined. The authors contacted the manufacturer after horse 1 was nebulized and due to the difficulties nebulizing the medication with the standard cup, it was determined that the fast cup would be more appropriate to use with this medication. The flow rate of the fast cup with saline was 2.5 mL/minute, and the volume mean diameter 50 (VMD 50) was 10 microns, thus 50% of the particles measured below 10 microns and 50% were higher. When the same cup was used with 10 mg/kg Vfend® the flow rate reduced to 0.4 mL/minute. In comparison, when used with 6.25 mg/mL of Vfend® the flow rate was 1.05 mL/min, and the VMD 50 was 6-7 microns, thus 50% of the particles were less than 6-7 microns and 50% were > 7 microns. If the medication was nebulized too fast and at a high concentration, the mesh cup created large size particles. Thus, the 6.25 mg/mL was considered to be the best concentration to use.

3.3.2 Clinical Feasibility of VRC Nebulization and VRC Concentrations in BALF and Plasma

The results are summarized in **Table 1**. VRC concentrations were undetectable in all plasma and BALF supernatant samples for all doses in all horses; further details about the delivery

and clinical feasibility are expanded below. Since VRC concentrations were below detection in BALF supernatant, urea dilution to calculate PELF concentrations was not performed. All horses tolerated the nebulization of VRC well (no coughing, snorting, head tossing, or flehmen responses), but became restless with long duration nebulization of some doses.

In horse 1, duration of nebulization was 90 minutes, and voriconazole concentrations in plasma, BALF supernatant and cell pellet were below detection at all timepoints **(Table 1).** Based on the duration of nebulization, the makers of the Flexineb® provided fast cups to improve the speed of nebulization, and the other doses of VFend® were tested using this type of cup.

Due to the lack of detectable concentrations of VRC in BALF after 200 mg, the dose was increased to 500 mg, but duration of nebulization was again very long at 115 minutes. VRC concentrations in plasma were below level of detection in all timepoints. Voriconazole concentrations obtained from the BALF cell pellet were detectable (0.4 µg/g) only at the end of nebulization **(Table 1)**, but below the MIC for *Aspergillus* spp., and concentrations in the BALF supernatant at all timepoints were below detection **(Table 1)**. There was no anti-fungal activity in the unseparated BALF fluid sample. After delivery of 500 mg, there were 20 mL of waste liquid in the aerosol chamber with a VRC concentration of 712.74 µg/mL, showing that 485 mg of the dose were delivered with 14.25 mg left in the chamber. For all other doses and horses, the fluid left in the chamber was very low (<500 µl), so VRC was not quantified in the residual.

For horses 3-6, the duration of nebulization was faster, but again, concentrations of VRC in plasma, BALF cell pellet and supernatant were below detection **(Table 1)**. There was no anti-fungal activity in unseparated BALF samples.

Bronchoalveolar lavage fluid samples collected after nebulization of all VRC dosages showed no cytological changes consistent with inflammation, and there were no significant differences in the percentage of neutrophils, lymphocytes, and macrophages between samples collected at screening sampling (within 2 weeks of nebulization) and samples collected after nebulization **(Table 1)**. Eosinophils and mast cells were considered very rare in all samples, so they were not quantified.

3.3.3 Voriconazole Concentrations after Nebulization in NPW

VRC concentrations were detectable in NPW samples at multiple timepoints and with multiple doses but were below 0.5 µg/mL by 6 hours in most horses. Horse 2 (nebulized with 500 mg) had VRC concentrations in the NPW at the end of nebulization of 47.4 µg/mL. Due to those very high concentrations and the long duration of nebulization (115 minutes), a lower dose of 100 mg was tested in horses 3 and 4, and the VRC concentrations in NPW samples were far above the MIC for *Aspergillus* spp. immediately post-nebulization, but concentrations dropped considerably by 1 and 6 hours post-nebulization **(Table 2).** In horses 5 and 6 (nebulized with 200 mg), the VRC concentrations in NPW samples were above the MIC for *Aspergillus* spp. immediately and at 1 hour post nebulization. At 6 hours, concentrations were below detection in horse 5, but right at 0.5 µg/mL in horse 6 **(Table 2)**, so an additional 5 horses were nebulized with 200 mg VRC to evaluate NPW concentrations up to 8 hours **(Table 2, Figure 4)**. In these 5 horses, duration of nebulization

was 38 ± 8 minutes. Plasma voriconazole concentrations were below detection at all timepoints. Mean VRC concentrations obtained from NPW samples immediately post-nebulization were significantly higher (*P*=0.0074) than at 8 hours, but other timepoints were not different **(Figure 4)**. Concentrations were below 0.5 µg/mL in most horses after 6 hours **(Figure 4)** and did not show antifungal activity.

1

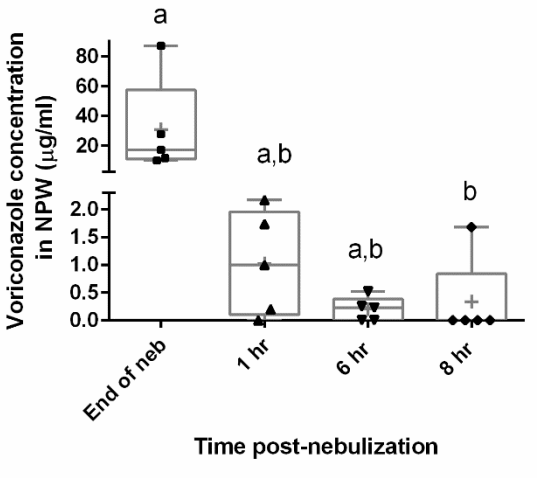

Figure 4: Voriconazole concentrations in nasopharyngeal wash samples (NPW) after nebulization of 200 mg VRC in 5 horses. Boxes represent the interquartile range, the whiskers are the minimum and maximum, the mean is a + sign, and the symbols are individual values. The dashed line represents the MIC for *Aspergillus* spp. at 0.5 µg/mL. Different letters are significantly different at P <0.05.

VRC dose (mg)	Horse ID	Duration of neb (min)	Time-point	Plasma [VRC] (µg/mL)	BALF [VRC]		Anti-fungal activity in BALF	NP in BALF (%)	Lymph in BALF (%)	MP in BALF (%)
					Cell pellet (µg/g)	SNT (µg/mL)				
200 (STD CUP)	1	90	SCRN	-	BD	BD	-	1	32	68
			0	BD	-	-	-	-	-	-
			EON	BD	-	-	-	-	-	-
			1 h PN	BD	BD	BD	-	0	45	55
			4 h PN	BD	-	-	-	-	-	-
			8 h PN	BD	BD	BD	-	-	-	-
500	2	115	SCRN	-	BD	BD	-	6	60	34
			0	BD	-	-	-	-	-	-
			EON	BD	0.40	BD	Neg	3	62	34
			2 h PN	BD	-	-	-	-	-	-
			6 h PN	BD	BD	BD	-	-	-	-
100	3	30	SCRN	-	BD	BD	-	3	44	53
			0	BD	-	-	-	-	-	-
			EON	BD	BD	BD	Neg	2	61	37
			1 h PN	BD	-	-	-	-	-	-
			6 h PN	BD	-	-	-	-	-	-
100	4	18	SCRN	-	BD	BD	-	9	60	31
			0	BD	-	-	-	-	-	-
			EON	BD	BD	BD	Neg	6	66	26
			1 h PN	BD	-	-	-	-	-	-
			6 h PN	BD	-	-	-	-	-	-
200	5	60	SCRN	-	BD	BD	-	*	*	*
			0	BD	-	-	-	-	-	-
			EON	BD	BD	BD	Neg	8	62	30
			1 h PN	BD	-	-	-	-	-	-
			6 h PN	BD	-	-	-	-	-	-
200	6	30	SCRN	-	BD	BD	-	10	40	50
			0	BD	-	-	-	-	-	-
			EON	BD	BD	BD	Neg	5	41	54
			1 h PN	BD	-	-	-	-	-	-
			6 h PN	BD	-	-	-	-	-	-

Table 1: Voriconazole (VRC) concentrations in BALF and plasma and cytological analysis before and after nebulization in each horse tested. All were nebulized with the fast cup except for horse 1, where indicated. The "–" indicates that samples were not collected or analyzed at that timepoint. Screening (SCRN) indicates initial BALF collected within 2 weeks of giving drug by nebulization. Time 0 is immediately before nebulization. (PN) post-nebulization; (BALF) bronchoalveolar lavage fluid; [48] end of nebulization; (neb) nebulization; (ID) identifier; (BD) below detection; (SNT) supernatant; [61] neutrophil; (Lymph) lymphocyte; [86] macrophage; [38] negative. *Low cellularity sample due to processing issue; cell population was normal but was not quantified.

VRC dose (mg)	Horse ID	Number of horses (n)	Timepoint	Plasma [VRC] (µg/mL)	NPW [VRC] (µg/mL)	Anti-fungal activity in NPW
500	2	1	0	BD	BD	-
			EON	BD	47.4	-
			2 h PN	BD	-	-
			6 h PN	BD	-	-
100	3	1	0	BD	BD	-
			EON	BD	19.9	-
			1 h PN	BD	1.31	-
			6 h PN	BD	0.66	-
100	4	1	0	BD	BD	-
			EON	BD	17.9	-
			1 h PN	BD	0.51	-
			6 h PN	BD	0.39	-
200	5	1	0	BD	BD	-
			EON	BD	5.77	-
			1 h PN	BD	1.15	-
			6 h PN	BD	BD	-
200	6	1	0	BD	BD	-
			EON	BD	16.5	-
			1 h PN	BD	2.81	-
			6 h PN	BD	2.23	-
200	#	5	0	*BD	BD	Neg
			EON	*BD	30.8 ± 29	Pos
			1 h PN	*BD	1.02 ± 0.84	-
			6 h PN	*BD	0.2 ± 0.19	Neg
			8 h PN	-	0.34 ± 0.67	-

Table 2: Voriconazole (VRC) concentrations in nasopharyngeal wash (NPW) and plasma after nebulization. Time 0 is immediately before nebulization. PN, post-nebulization; ID, identifier; BD, below detection; EON, end of nebulization; Neg, negative; Pos, positive. #Additional group of 5 horses that only had NPW and plasma samples collected and analyzed. The "–" indicates that samples were not collected or analyzed at that timepoint. *Plasma values were BD in all 5 horses.

3.4 Discussion

Nebulization of sterile VRC solution (Vfend®) using a common, commercial nebulizer (Flexineb®) did not achieve adequate concentrations of VRC in the lower airways at the doses tested (100-500 mg of VRC), so delivery of Vfend® by this method is unlikely to be clinically useful for treatment of pneumomycosis. This is important information for equine practitioners that may encounter fungal pneumonia cases and have access to a Flexineb® nebulizer in their practice. Detectable concentrations of VRC were found in the upper airway using this delivery mechanism, and further study is needed to determine dosage interval for clinical applicability in upper respiratory fungal infections.

The initial dose tested of 200 mg was based on human[75] and murine[55] studies, but did not result in detectable BALF drug concentrations using this nebulizer. Increasing the dose to 500 mg and using the fastest delivery cup available for this nebulizer still did not achieve adequate concentrations in BALF, and the duration of nebulization (almost 2 hours) was not clinically practical. The clinical significance of very low concentrations was reinforced in this study by the lack of antifungal activity from BALF samples evaluated via the agar disk diffusion method.

The low BALF concentrations could have been impacted by multiple factors. Vfend® solution for injection was not easy to nebulize and required 18 to 115 minutes (depending on the amount given and cup used) for delivery. The difficulty in nebulizing was likely due to the physical characteristics of the medication, such as low water solubility and high viscosity. Vfend® contains sulfobutyl ether-β-cyclodextrin, an excipient that should improve the water solubility of the medication, but that did not improve the ability to nebulize this preparation. High viscosity drugs,

or those with large particle size, may not nebulize well as they may clog the small holes of the membrane within the medication cup. For this reason, after the first horse was nebulized with a standard cup and analysis was performed by the nebulizer manufacturer, the fast cup was used with the rest of the horses. The clogging also explains why the high dose of 500 mg had so much residual volume left in the cup and explains some of the variability in duration of nebulization despite the same volume of drug administration. Strict cleaning of the cup according to manufacturer's recommendations or replacement of the cups frequently would be required for clinical use of these cups with Vfend® since more difficult to nebulize medications can decrease the lifespan of the cups.

In addition, the VMD 50 for this nebulizer and using the fast cup showed a particle size that may have been too large for a therapeutic amount of drug to reach the alveoli and be subsequently absorbed. A previous report in horses[59] determined that the particle size needed to reach the alveoli for absorption should measure between 1 - 5 µm. The particle size obtained in this study had a VMD 50 of 6-7 µm, meaning that 50% of the particles were that size (6 - 7 µm) or greater, and 50% of the particles were that size or smaller; thus, the amount of medication that reached the alveoli was likely lower than ideal.

Further, as the dose elected was determined based on similar murine[55] and human[75] studies using nebulized Vfend® solution and previous equine studies that used orally administered VRC[51,52], it is possible that the dose was lower than the dose needed to detect VRC in the BALF. The pharmacokinetics of the drug could have also contributed to the low VRC concentrations in plasma and BALF samples. Based on a human study[47], this drug shows rapid absorption and

nonlinear pharmacokinetics due to its capacity-limited elimination, and thus it is considered a dose dependent medication. The volume of distribution is high (2 - 4.6 L/kg), thus indicating an extensive distribution into extracellular and intracellular compartments. Lung biopsies with measurement of drug in the tissues would be required to investigate this mechanism, but does not seem warranted based on not even finding detectable drug immediately after nebulization in the BALF supernatant or cell pellet of most doses.

When VRC is administered orally in people, a loading dose is recommended to reach plasma concentrations close to steady state within the first 24 hours of dosing. Without the loading dose, the accumulation occurs after multiple-dosing with steady-state plasma VRC concentrations being achieved by day 6[48]. In a murine study[55,87], a high concentration of VRC was achieved following multiple inhaled doses of VRC solution. In the present study, the horses were nebulized only once and the concentrations of VRC were undetectable in the BALF supernatant and plasma, which may indicate that multiple doses per day may be necessary to obtain higher VRC concentrations in the BALF and plasma. Costs of drugs change frequently and should not preclude investigation, but at current rates, 200 mg of Vfend® is $60-$80 USD, so higher and more frequent doses may not be cost effective for many clinical cases in some parts of the world for the duration of treatment that is typically needed for pneumomycosis.

After determining that Vfend® delivery by this method would not be feasible for treatment of fungal pneumonia, we investigated VRC deposition in the upper airways. VRC concentrations in NPW samples immediately after nebulization of 100 mg, 200 mg, and 500 mg of VRC were well over the MIC for *Aspergillus* spp. ($\geq$ 0.5 μg/mL), but generally decreased to below the MIC

by 6 hours. Fungal infections of the URT, such as fungal rhinitis and guttural pouch mycosis (which is commonly caused by *Aspergillus* spp.) are uncommon but occur more frequently than pneumomycosis, and treatment remains challenging[88,89]. Nebulized VRC may introduce a new treatment option for fungal diseases affecting the URT that could have the benefits of reaching a higher concentration at the site of infection and decreasing the incidence of side effects associated with systemic therapy[37,90]. Results from the current study could provide a framework to further investigate appropriate dose and frequency of VRC nebulization for treatment of URT fungal infections. More research is needed to make clinical dosing recommendations, such as measuring the VRC concentrations reached in the paranasal sinuses and guttural pouches and determination of an appropriate dose and dose interval since samples were not collected between 1- and 6-hours post-nebulization. It may be that a lower dose can be given more frequently. Also, as mentioned previously, it is possible that multiple daily dosing via nebulization may yield higher concentrations in the upper airways. The same cost limitations as described above could apply in some cases.

A central component of the primary objective of this study was to determine whether concentrations of VRC in PELF achieved MIC levels for *Aspergillus* sp. The PELF is a thin layer of fluid that covers the epithelium of the small airways and alveoli and drug concentrations within the PELF are thought to best predict antimicrobial activity against extracellular pathogens associated with pneumonia[91,92]. When a BAL is performed, the PELF becomes diluted within the BALF and thus a dilution correction must be performed to calculate the actual PELF drug concentration. Urea is most often used as an endogenous marker for this dilution correction where it is assumed that the urea concentration within the PELF approximates urea concentration within

the plasma[91,93,94]. It is this relationship that allows for calculation of the volume of PELF within the BALF volume. Once the PELF volume is determined, the drug concentration within the PELF can be derived from the measured drug concentration within the BALF. Thus determination of PELF drug concentration relies on achieving detectable drug concentrations within BALF. This method has been described in several species for a variety of antimicrobials, and although not without limitations[95,96], it is considered the method of choice for determining PELF drug concentration. In this study, because the concentrations of VRC were below the limit of detection within the BALF for all horses, urea dilution was not performed. Similar dilution methods have not been validated or described for fluids obtained from upper airway washes in horses, thus although drug concentrations were detected within the NPW samples, we did not perform urea dilution. For the BAL procedures and for the NPW, a similar volume of fluid was obtained from each horse after the wash, which should reduce the variability in the lining fluid dilution between samples.

This study has limitations that need consideration. The results obtained in this study can only be applied to the Flexineb®, and other nebulizers such as those used in human studies (e.g. jet nebulizers) could be more effective at delivering this particular medication; however, this would require further study that was beyond the scope of this project. The small group of horses included in the study and the lack of correction for dilution of the NPW could have affected the variable drug concentrations obtained in the NPW fluid.

3.5 Conclusions

The concentrations of VRC after nebulization of Vfend® solution with a Flexineb® nebulizer in plasma and BALF samples were clinically insignificant at all timepoints, so delivery of Vfend® by this method is not recommended for treatment of fungal pneumonia in horses. The concentrations and antifungal activity in NPW samples suggest that nebulization of Vfend® solution may be more beneficial when treating upper respiratory tract fungal infections; however, further investigation into dose and dosing frequency is needed before this can be recommended.

Chapter 4 – Summary and Conclusions

Fungal infections of the respiratory tract in horses are considered rare, with the upper airways being more frequently affected compared to the lower airways and lungs. Because diagnosis and treatment of such infections are difficult, the prognosis is typically guarded to poor. Furthermore, the available drugs to treat fungal respiratory disease in horses are limited, often cost-prohibited, and may cause important side effects. Inhalation therapy is an alternative route to deliver higher drug concentrations to the lower airways, decrease the dose of medication used and avoid or decrease their systemic effects.

The use of the Flexineb® nebulizer for delivery of voriconazole (Vfend® solution) in this study demonstrated concentrations of voriconazole in the upper respiratory tract above the target MIC (≥ 0.5 μg/ml) for the most common fungi implicated in equine respiratory fungal infections, such as *Aspergillus* spp. These high concentrations were reached as soon as 30 minutes post-nebulization after nebulization of 200 mg of Vfend® solution and maintained for up to 6 hours. However, detectable concentrations could not be measured in the lower airways when administered the same dosage.

This study has demonstrated that nebulization of Vfend® solution in horses is not clinically useful to deliver this particular drug to the lower airways effectively. This was determined based on the duration of nebulization and the low drug concentration obtained in BAL samples after nebulization. The difficulty in delivering VRC solution using the Flexineb® nebulizer was suspected to be due to the physical characteristics of the medication, such as the viscosity, and the

nebulizer unit itself. Evaluation of the particle size achieved in this study with the use of the Flexineb® nebulizer and a fast cup when administering Vfend® solution, showed that approximately 50% of particles attained were of a large size, which affected the deposition of the drug in the lower airways as larger particles are more likely to be trapped in the URT or eliminated by the mucociliary apparatus and do not reach the lower airways and lungs to be absorbed.

Results from this study are of potential interest for the treatment of upper respiratory fungal infection in horses with future applications to other animal species. The next step to better understand the delivery of this drug via nebulization and the effects in the upper respiratory tract would be to use this protocol in a larger number of healthy horses and determine a dose and dosing interval, measure the concentration of drug achieved within different tissues (nasal, paranasal, and guttural pouch mucosa), and assess case outcome and treatment cost.

In conclusion, the delivery of Vfend® solution via nebulization is not recommended for treatment of pneumomycosis in adult horses but may be more beneficial when treating upper respiratory tract fungal infections.

References

1. Robinson EN, Furlow PW. Anatomy of the Respiratory System In: McGorum BC, Dixon PM, Robinson EN, et al., eds. *Equine Respiratory Medicine and Surgery*. Philadelphia, PA, USA: Saunders, 2007;3-17.

2. Burton A, Giguère S, Arnold R. Pharmacokinetics, pulmonary disposition and tolerability of liposomal gentamicin and free gentamicin in foals. *Equine veterinary journal* 2015;47:467-472.

3. Kumar P, Timoney J, Southgate H, et al. Light and scanning electron microscopic studies of the nasal turbinates of the horse. *Anat Histol Embryol* 2000;29:103-109.

4. Baptiste KE, Naylor JM, Bailey J, et al. A function for guttural pouches in the horse. *Nature* 2000;403:382-383.

5. Yu M, Wang Z, Robinson N, et al. Inhibitory nerve distribution and mediation of NANC relaxation by nitric oxide in horse airways. *Journal of Applied Physiology* 1994;76:339-344.

6. Broadstone R, LeBlanc P, Derksen F, et al. In vitro responses of airway smooth muscle from horses with recurrent airway obstruction. *Pulm Pharmacol* 1991;4:191-202.

7. McLaughlin RF, Tyler WS, Canada RO. A study of the subgross pulmonary anatomy in various mammals. *Am J Anat* 1961;108:149-165.

8. Aijaz S, Balda MS, Matter K. Tight junctions: molecular architecture and function. *Int Rev Cytol* 2006;248:261-298.

9. Chai J, Long B, Liu X, et al. Effects of sevoflurane on tight junction protein expression and PKC-α translocation after pulmonary ischemia–reperfusion injury. *Exp Mol Med* 2015;47:e167-e167.

10. Davis E. Disorders of the Respiratory System In: Reed SM, Bayly WM,Sellon DC, eds. *Equine Internal Medicine*. St. Louis, Missouri: Elsevier, 2018;313-386.

11. Davis EG, Freeman DE, Hardy J. Respiratory Infections In: Sellon DC,Long MT, eds. *Equine Infectious Diseases*. Second ed. St. Louis, Missouri: Elsevier, 2014;1-21.

12. Mealey RH, Long MT. Mechanisms of Disease and Immunity In: Reed SM, Warwick M. B,Sellon DC, eds. *Equine Internal Medicine*. 4th ed. St Louis, MI: Elsevier, 2018;3-78.

13. Cafarchia C, Figueredo LA, Otranto D. Fungal diseases of horses. *Veterinary microbiology* 2013;167:215-234.

14. H. MR, Long MT. Mechanisms of Disease and Immunity In: Reed SM, Bayly WM,Sellon DC, eds. *Equine Internal Medicine*. 4th ed. St. Louis, Missouri: Elsevier, 2018;3-78.

15. Stewart AJ, Cuming RS. Update on Fungal Respiratory Disease in Horses. *Veterinary Clinics: Equine Practice* 2015;31:43-62.

16. Tell LA. Aspergillosis in mammals and birds: impact on veterinary medicine. *Medical Mycology* 2005;43:S71-S73.

17. Sellon DC, Kohn C. Aspergillosis In: Sellon DC,Long MT, eds. *Equine Infectious Diseases (Second Edition)*. St. Louis: W.B. Saunders, 2014;421-433.e424.

18. Zmeili OS, Soubani A. Pulmonary aspergillosis: a clinical update. *Journal of the Association of Physicians* 2007;100:317-334.

19. Groll A, Shah P, Mentzel C, et al. Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. *J Infect* 1996;33:23-32.

20. Slocombe R, Slauson D. Invasive pulmonary aspergillosis of horses: an association with acute enteritis. *Veterinary pathology* 1988;25:277-281.

21. Pirie R, Dixon P, McGorum B. Endotoxin contamination contributes to the pulmonary inflammatory and functional response to Aspergillus fumigatus extract inhalation in heaves horses. *Clin Exp Allergy* 2003;33:1183-1189.

22. Clarke A. Air hygiene and equine respiratory disease. *In Pract* 1987;9:196-204.

23. Webster A, Clarke A, MADELIN TM, et al. Air hygiene in stables 1: Effects of stable design, ventilation and management on the concentration of respirable dust. *Equine veterinary journal* 1987;19:448-453.

24. Sweeney C, Habecker P. Pulmonary aspergillosis in horses: 29 cases (1974-1997). *J Am Vet Med Assoc* 1999;214:808-811.

25. Stewart AJ, Welles EG, Salazar T. Fungal infections of the upper respiratory tract. *Compendium Equine* 2008;3:208-220.

26. Couëtil LL, Hawkins J. Diagnostic tests and therapeutic procedures. *Respiratory diseases of the horse: A problem-oriented approach to diagnosis & management*. London, UK: Manson Publishing Ltd., 2013;47-76.

27. Bašková L, Buchta V. Laboratory diagnostics of invasive fungal infections: an overview with emphasis on molecular approach. *Folia Microbiol (Praha)* 2012;57:421-430.

28. White PL, Archer AE, Barnes RA. Comparison of non-culture-based methods for detection of systemic fungal infections, with an emphasis on invasive Candida infections. *J Clin Microbiol* 2005;43:2181-2187.

29. Mengoli C, Cruciani M, Barnes RA, et al. Use of PCR for diagnosis of invasive aspergillosis: systematic review and meta-analysis. *The Lancet infectious diseases* 2009;9:89-96.

30. Sellon DC, Long MT. Laboratory diagnosis of fungal diseases In: Byrne BA, ed. *Respiratory Diseases of the Horse*. Second ed. St. Louis, Missouri: Saunders Elsevier, 2014;393-399.el.

31. Guillot J, Sarfati J, De Barros M, et al. Comparative study of serological tests for the diagnosis of equine aspergillosis: British Medical Journal Publishing Group, 1999.

32. Higgins JC, Pusterla N, Pappagianis D. Comparison of Coccidioides immitis serological antibody titres between forms of clinical coccidioidomycosis in horses. *The Veterinary Journal* 2007;173:118-123.

33. Freeman DE. Update on disorders and treatment of the guttural pouch. *Veterinary Clinics: Equine Practice* 2015;31:63-89.

34. Hilton H, Galuppo L, Puchalski S, et al. Successful treatment of invasive pulmonary aspergillosis in a neonatal foal. *J Vet Intern Med* 2009;23:375-378.

35. Nappert G, Van Dyck T, Papich M, et al. Successful treatment of a fever associated with consistent pulmonary isolation of Scopulariopsis sp. in a mare. *Equine veterinary journal* 1996;28:421-424.

36. Begg L, Hughes K, Kessell A, et al. Successful treatment of cryptococcal pneumonia in a pony mare. *Australian veterinary journal* 2004;82:686-692.

37. Higgins JC, Pusterla N. Fungal pneumonia in horses. *Clinical Techniques in Equine Practice* 2006;5:218-224.

38. Tolman JA, Wiederhold NP, McConville JT, et al. Inhaled voriconazole for prevention of invasive pulmonary aspergillosis. *Antimicrobial therapy in veterinary medicine* 2009;53:2613-2615.

39. Hilberg O, Andersen CU, Henning O, et al. Remarkably efficient inhaled antifungal monotherapy for invasive pulmonary aspergillosis. *European Respiratory Journal* 2012;40:271-273.

40. Hilberg O, Bendstrup E, Mortensen J, et al. Inhaled voriconazole-an efficient treatment against invasive aspergillosis. *D63 ADVANCES IN TREATMENT OF RESPIRATORY INFECTIONS*: American Thoracic Society, 2012;A6088-A6088.

41. Le J, Schiller DS. Aerosolized delivery of antifungal agents. *Current fungal infection reports* 2010;4:96-102.

42. Chan H. Pharmacokinetics of Voriconazole in Horses and Alpacas, 2008.

43. Davis PR, Meyer GA, Hanson RR, et al. Pseudallescheria boydii infection of the nasal cavity of a horse. *Journal of the American Veterinary Medical Association* 2000;217:707-709.

44. Davis JL, Salmon JH, Papich MG. Pharmacokinetics and tissue distribution of itraconazole after oral and intravenous administration to horses. *American journal of veterinary research* 2005;66:1694-1701.

45. Merritt A. The equine stomach: a personal perspective (1963-2003). Proceedings of the 49th Annual Convention of the American Association of Equine Practitioners, New Orleans, Louisiana, USA, 21-25 November 2003 2003;75-102.

46. Herbrecht R, Denning DW, Patterson TF, et al. Voriconazole versus Amphotericin B for Primary Therapy of Invasive Aspergillosis. *New England Journal of Medicine* 2002;347:408-415.

47. Theuretzbacher U, Ihle F, Derendorf H. Pharmacokinetic/Pharmacodynamic Profile of Voriconazole. *Clin Pharmacokinet* 2006;45:649-663.

48. Saravolatz LD, Johnson LB, Kauffman CA. Voriconazole: A New Triazole Antifungal Agent. *Clinical Infectious Diseases* 2003;36:630-637.

49. Giguère S. Antifungal chemotherapy. *Antimicrobial therapy in veterinary medicine* 2006;4:301-322.

50. Colitz CM, Latimer FG, Cheng H, et al. Pharmacokinetics of voriconazole following intravenous and oral administration and body fluid concentrations of voriconazole following repeated oral administration in horses. *American journal of veterinary research* 2007;68:1115-1121.

51. Passler NH, Chan HM, Stewart AJ, et al. Distribution of voriconazole in seven body fluids of adult horses after repeated oral dosing. *J Vet Pharmacol Ther* 2010;33:35-41.

52. Davis JL, Salmon JH, Papich MG. Pharmacokinetics of voriconazole after oral and intravenous administration to horses. *American Journal of Veterinary Research* 2006;67:1070-1075.

53. Quimby J, Hoffman S, Duke J, et al. Adverse neurologic events associated with voriconazole use in 3 cats. *J Vet Intern Med* 2010;24:647-649.

54. Erwin GE, Fitzgerald JE. Case report: allergic bronchopulmonary aspergillosis and allergic fungal sinusitis successfully treated with voriconazole. *J Asthma* 2007;44:891-895.

55. Tolman JA, Nelson NA, Son YJ, et al. Characterization and pharmacokinetic analysis of aerosolized aqueous voriconazole solution. *European Journal of Pharmaceutics and Biopharmaceutics* 2009;72:199-205.

56. Holle J, Leichsenring M, Meissner P. Nebulized voriconazole in infections with Scedosporium apiospermum—Case report and review of the literature. *Journal of Cystic Fibrosis* 2014;13:400-402.

57. Mazan MR, Ceresia ML. Clinical pharmacology of the respiratory system In: Cole C, Bentz B,Maxwell L, eds. *Equine pharmacology*. Ames, IO: Wiley Blackwell, 2015;139-182.

58. Rodvold KA, George JM, Yoo L. Penetration of anti-infective agents into pulmonary epithelial lining fluid. *Clin Pharmacokinet* 2011;50:637-664.

59. Cha ML, Costa LR. Inhalation Therapy in Horses. *Veterinary Clinics: Equine Practice* 2017;33:29-46.

60. Kuhn RJ. Pharmaceutical considerations in aerosol drug delivery. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* 2002;22:80S-85S.

61. Duvivier DH, Votion D, Vandenput S, et al. Aerosol therapy in the equine species. *Vet J* 1997;154:189-202.

62. Labiris N, Dolovich M. Pulmonary drug delivery. Part I: physiological factors affecting therapeutic effectiveness of aerosolized medications. *Br J Clin Pharmacol* 2003;56:588-599.

63. Lourenço RV, Cotromanes E. Clinical aerosols: I. Characterization of aerosols and their diagnostic uses. *Arch Intern Med* 1982;142:2163-2172.

64. McKenzie HC. Characterization of antimicrobial aerosols for administration to horses. *Veterinary therapeutics: research in applied veterinary medicine* 2003;4:110.

65. Finney MJ, Anderson SD, Black JL. The effect of non-isotonic solutions on human isolated airway smooth muscle. *Respir Physiol* 1987;69:277-286.

66. Duvivier DH, Votion D, Roberts C, et al. Inhalation therapy of equine respiratory disorders. *Equine Veterinary Education* 1999;11:124-131.

67. Bullone M, Vargas A, Elce Y, et al. Fluticasone/salmeterol reduces remodelling and neutrophilic inflammation in severe equine asthma. *Sci Rep* 2017;7:1-13.

68. de Pablo E, Fernández-García R, Ballesteros MP, et al. Nebulised antibiotherapy: conventional versus nanotechnology-based approaches, is targeting at a nano scale a difficult subject? *Annals of translational medicine* 2017;5.

69. Hoffman AM. Inhaled Medications and Bronchodilator Usage in the Horse. *Veterinary Clinics of North America: Equine Practice* 1997;13:519-530.

70. Votion D, Ghafir Y, Munsters K, et al. Aerosol deposition in equine lungs following ultrasonic nebulisation versus jet aerosol delivery system. *Equine veterinary journal* 1997;29:388-393.

71. Olsson B, Bondesson E, Borgström L, et al. Pulmonary Drug Metabolism, Clearance, and Absorption In: Smyth HDC,Hickey AJ, eds. *Controlled Pulmonary Drug Delivery*. New York, NY: Springer New York, 2011;21-50.

72. Lombry C, Edwards DA, Préat V, et al. Alveolar macrophages are a primary barrier to pulmonary absorption of macromolecules. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 2004;286:L1002-L1008.

73. Oberdörster G. Lung clearance of inhaled insoluble and soluble particles. *J Aerosol Med* 1988;1:289-330.

74. Anttila S, Hukkanen J, Hakkola J, et al. Expression and localization of CYP3A4 and CYP3A5 in human lung. *Am J Respir Cell Mol Biol* 1997;16:242-249.

75. Andersen CU, Sønderskov LD, Bendstrup E, et al. Voriconazole Concentrations in Plasma and ELF after Inhalation and Oral Treatment. *Basic & Clinical Pharmacology & Toxicology* 2017.

76. Tolman JA, Wiederhold NP, McConville JT, et al. Inhaled voriconazole for prevention of invasive pulmonary aspergillosis. *Antimicrobial agents and chemotherapy* 2009;53:2613-2615.

77. Arora S, Haghi M, Loo C-Y, et al. Development of an inhaled controlled release voriconazole dry powder formulation for the treatment of respiratory fungal infection. *Molecular pharmaceutics* 2015;12:2001-2009.

78. Howard A, Hoffman J, Sheth A. Clinical application of voriconazole concentrations in the treatment of invasive aspergillosis. *Annals of Pharmacotherapy* 2008;42:1859-1864.

79. Pirie RS, McGorum BC. Inhalation therapy for equine lower respiratory tract disease. *In Pract* 2017;39:317-327.

80. Fultz L, Giguère S, Berghaus LJ, et al. Pulmonary pharmacokinetics of desfuroylceftiofur acetamide after nebulisation or intramuscular administration of ceftiofur sodium to weanling foals. *Equine veterinary journal* 2015;47:473-477.

81. PA. W. Performance Standards for Antifungal Disk Diffusion Susceptibility Testing of Nondermatophyte Filamentous Fungi; Informational Supplement. CLSI Document M51-S1. *Clinical and Laboratory Standards Institute* 2010.

82. Wasko A, Barkema H, Nicol J, et al. Evaluation of a risk-screening questionnaire to detect equine lung inflammation: results of a large field study. *Equine veterinary journal* 2011;43:145-152.

83. Couëtil LL, Hoffman AM, Hodgson J, et al. Inflammatory airway disease of horses. *J Vet Intern Med* 2007;21:356-361.

84. Cuming RS, Abarca EM, Duran S, et al. Development of a Sustained-Release Voriconazole-Containing Thermogel for Subconjunctival Injection in Horses. *Investigative Ophthalmology & Visual Science* 2017;58:2746-2754.

85. Alastruey-Izquierdo A. MM, Bonfietti LX., Rodriguez-Tudela JL. Susceptibility Test for Fungi: Clinical and Laboratorial Correlations in Medical Mycology. *Rev Inst Med Trop Sao Paulo* 2015;57:57-64.

86. Scott LJ, Simpson D. Voriconazole. *Drugs* 2007;67:269-298.

87. Tolman JA, Nelson NA, Bosselmann S, et al. Dose tolerability of chronically inhaled voriconazole solution in rodents. *International journal of pharmaceutics* 2009;379:25-31.

88. Tremaine W, Dixon P. A long-term study of 277 cases of equine sinonasal disease. Part 1: details of horses, historical, clinical and ancillary diagnostic findings. *Equine veterinary journal* 2001;33:274-282.

89. Ludwig A, Gatineau S, Reynaud M-C, et al. Fungal isolation and identification in 21 cases of guttural pouch mycosis in horses (1998–2002). *The Veterinary Journal* 2005;169:457-461.

90. Korenek NL, Legendre AM, Andrews FM, et al. Treatment of mycotic rhinitis with itraconazole in three horses. *J Vet Intern Med* 1994;8:224-227.

91. Giguère S, Huang R, Malinski TJ, et al. Disposition of gamithromycin in plasma, pulmonary epithelial lining fluid, bronchoalveolar cells, and lung tissue in cattle. *American journal of veterinary research* 2011;72:326-330.

92. Kiem S, Schentag JJ. Interpretation of antibiotic concentration ratios measured in epithelial lining fluid. *Antimicrobial agents and chemotherapy* 2008;52:24-36.

93. Rennard S, Basset G, Lecossier D, et al. Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. *Journal of Applied Physiology* 1986;60:532-538.

94. Kirschvink N, Fiévez L, Dogné S, et al. Comparison of inulin with urea as dilutional markers of bronchoalveolar lavage in healthy and heaves-affected horses. *Vet Res* 2001;32:145-154.

95. Melamies MA, Järvinen A-K, Seppälä KM, et al. Comparison of results for weight-adjusted and fixed-amount bronchoalveolar lavage techniques in healthy Beagles. *American journal of veterinary research* 2011;72:694-698.

96. Marcy TW, Merrill WW, Rankin JA, et al. Limitations of using urea to quantify epithelial lining fluid recovered by bronchoalveolar lavage. *Am Rev Respir Dis* 1987;135:1276-1280.