Chapter 4 Reproducibility of endoscopic grading using tracheobronchoscopy in racehorses

4.1 ABSTRACT

We determined the interobserver reliability for the assessment of exercise-induced pulmonary haemorrhage (EIPH), pharyngeal lymphoid hyperplasia (PLH), arytenoid cartilage movement (ACM) and tracheal mucous (TM) following tracheobronchoscopic examination in 1,011 Thoroughbred racehorses. Tracheobronchoscopic examinations were performed on racehorses < 2 hours after racing and recorded onto digital video disc. Three veterinarians then assessed all recordings independently for the presence and severity of EIPH, PLH, ACM, and TM. All scores were tabulated and concordance was measured using the weighted κ statistic (κ_w).

The interobserver agreement was the highest for EIPH (κ_w = 0.78 to 0.84) and moderate for PLH (κ_w = 0.43 to 0.52), ACM (κ_w = 0.43 to 0.56) and TM (κ_w = 0.43 to 0.57). All observers agreed or 2 of 3 agreed and the third differed by ≤ 1 grade in 99.6% of observations for EIPH, 98.29% of observations for PLH, 100% of observations for ACM, and 91.67% of observations for TM.

Although the interobserver reliability of tracheobronchoscopic evaluation of EIPH was the highest as compared with PLH, ACM and TM; all four classifications are sufficiently reproducible when used by veterinarians performing tracheobronchoscopic examinations on horses.

4.2 INTRODUCTION

Risk factors for poor performance in Thoroughbred racehorses may include exercise-induced pulmonary haemorrhage (EIPH),[5] tracheal mucous (TM),[7] and idiopathic laryngeal hemiplegia (ILH).[8] Antigenic stimulation of the nasopharynx may cause localized inflammation of the nasopharynx resulting in pharyngeal lymphoid hyperplasia (PLH). Although PLH is not associated with impaired racing performance,[7] it may predispose to upper airway obstruction,[9] thereby negatively affecting performance.

Health and athleticism of racehorses may be affected by EIPH, TM, ILH and PLH and therefore reliable, repeatable methods of assessment are needed. Such methods should be able to accurately and quickly assess the severity of the condition and be able to monitor efficacy of treatment once therapy has started. Tracheobronchoscopy is a quick, minimally-invasive technique without laborious, time-consuming laboratory processing of samples that allows immediate classification of racehorses according to previously established grading systems for EIPH,[6] TM,[3] arytenoid cartilage movement (ACM)[12] and PLH.[1] Although the repeatability of interobserver reliability of tracheobronchoscopic

assessment of EIPH in Thoroughbred racehorses has been reported before,[6] no reports on the interobserver reliability for the detection of TM, ACM and PLH exist.

We sought to determine the interobserver reliability of tracheobronchoscopic assessment of the presence and severity of EIPH, PLH, ACM, and TM in Thoroughbred racehorses in South Africa.

4.3 MATERIALS AND METHODS

4.3.1 Thoroughbred racehorses

Tracheobronchoscopic examinations were performed on 1,011 Thoroughbred racehorses < 2 hours after racing at 5 race venues and in 28 race meets. Races were 800 to 3,200 meter flat races run on turf or sand at Turffontein (Gauteng Province) and Vaal (Free State Province) Race Courses; and at sea level at Clairwood and Greyville Turf Clubs (Kwazulu-Natal Province) and Kenilworth Race Course (Western Cape Province) in South Africa from August 4 to December 19, 2005.

4.3.2 Endoscopic examination

Following racing, unsedated racehorses were restrained by the use of a halter and nose twitch in a dedicated examination stable. Tracheobronchoscopic (Pentax Corporation, Tokyo, Japan) evaluation of the nasopharynx, larynx and trachea to the level of the carina

took place and all examinations were recorded onto digital video disc. All recordings were then independently reviewed by 3 veterinarians.

4.3.3 Grading of EIPH, ACM, PLH and TM

Racehorses were graded 0 to 4 for EIPH.[6] Briefly, grade 0 indicated the absence of blood in the pharynx, larynx, trachea, or mainstem bronchi; grade 1 indicated the presence of 1 or more flecks of blood or ≤ 2 short (< $^1/_4$ length of the trachea), narrow (< 10% of the tracheal surface area) streams of blood in the trachea or mainstem bronchi (Figure 2.1); grade 2 indicated long stream of blood (> $^1/_2$ length of the trachea) or > 2 short streams covering < $^1/_3$ of the tracheal circumference (Figure 2.2); grade 3 indicated multiple, distinct streams of blood covering > $^1/_3$ of the tracheal circumference without blood pooling at the thoracic inlet (Figure 2.3); and grade 4 indicated multiple, coalescing streams of blood covering > 90% of the tracheal surface with blood pooling at the thoracic inlet (Figure 2.4).

Mucous within the trachea was graded 0 to 5.[3] Grade 0 indicated the absence of mucous; grade 1 indicated singular droplets of mucous (Figure 3.8); grade 2 indicated multiple droplets of mucous that is partly confluent (Figure 3.9); grade 3 indicated mucous that is ventrally confluent (Figure 3.10); grade 4 indicated a large ventral pool of mucous (Figure 3.11); and grade 5 indicated profuse amounts of mucous covering > 25% of the tracheal lumen (Figure 3.12).

The presence of ILH was assessed through severity of ACM and was graded 1 to 4.[12] Grade 1 indicated symmetrical synchronous abduction and adduction of the left and right arytenoid cartilages (Figure 3.1); grade 2 indicated some asynchronous movement (hesitation, flutter or abductor weakness) of the left arytenoid cartilage during any phase of respiration and full abduction of the left arytenoid cartilage which could be maintained by swallowing or nasal occlusion; grade 3 indicated asynchronous movement (hesitation, flutter or abductor weakness) of the left arytenoid cartilage during any phase of respiration and full abduction of the left arytenoid cartilage could not be induced or maintained by swallowing or nasal occlusion, and grade 4 indicated no substantial movement of the left arytenoid cartilage during any phase of respiration and were subsequently classified as having ILH (Figure 3.2).

The severity of PLH was graded on a scale from 1 to 4.[1] Grade 1 indicated lymphoid hyperplasia limited to < 180° of the dorsal pharyngeal recess (Figure 3.3); grade 2 indicated lymphoid hyperplasia extending to circumference of the dorsal pharyngeal recess (Figure 3.4); grade 3 indicated lymphoid hyperplasia made midline contact of the dorsal pharyngeal recess (Figure 3.5); and grade 4 indicated small masses (which may be abscesses) arising from either the dorsal pharyngeal recess or the pharyngeal walls (Figure 3.6).

4.3.4 Data analysis

Weighted kappa statistics (κ_w) were calculated for each combination of observers for the grading of EIPH, PLH, ACM, and TM. Partial agreement can be taken into account using a weighted kappa in which the pairs of test results that are close are considered to be in partial agreement through the use of a weight matrix. The weighted matrix used for the kappa statistic was one of the prerecorded matrixes (w) used by STATA (STATA Statistical Software [release 8]: STATA Corporation, College Station, Texas, USA).The weights are given by $1-|i-j| / (k-1)$, where i and j index the rows of columns of the ratings by the two raters and k is the maximum number of possible ratings. The weightings (agreement) used for EIPH and ACM if there was one rating apart was 0.75, two ratings apart was 0.5, three ratings apart was 0.25 and > three was 0. The weightings for PLH were 0.6667, 0.3333 and 0 for 1, 2 and 3 ratings apart respectively. The weightings for TM were 0.8, 0.6, 0.4, 0.2 and 0 for 1 to 5 ratings apart respectively. The strength of agreement was considered poor ($\kappa_w < 0.20$), fair ($\kappa_w = 0.21$ to 0.40), moderate ($\kappa_w = 0.41$ to 0.60), good ($\kappa_w = 0.61$ to 0.80) and very good ($\kappa_w = 0.81$ to 1.00).[9] Mean results with upper and lower 95% confidence interval (CI) are reported.

4.4 RESULTS

Good to very good interobserver reliability was reported for EIPH ($\kappa_w = 0.78$, [95% CI: 0.74 to 0.82]; $\kappa_w = 0.83$ [95% CI: 0.79 to 0.88]; and $\kappa_w = 0.84$ [95% CI: 0.80 to 0.88]). Agreement between the 3 reviewers was observed for 386 examinations as grade 0, 222

as grade 1, 50 as grade 2, 41 as grade 3, and 20 as grade 4. Complete agreement between the 3 observers was present in 71.1% of all examinations. Scores of 2 of 3 observers agreed and that of the third observer differed by ≥ 1 grade in 28% of examinations. All three observers disagreed in 0.4% (n = 4) of observations. All observers agreed or 2 of 3 agreed and the third observer differed by ≤ 1 grade in 99.6% of observations.

Moderate inter-observer reliability was reported for PLH (κ_w = 0.43 [95% CI: 0.37 to 0.48]; κ_w = 0.46 [95% CI: 0.41 to 0.51]; and κ_w = 0.52 [95% CI: 0.47 to 0.57]). Agreement between the 3 reviewers was observed for 233 examinations as grade 1, 310 examinations as grade 2, 6 examinations as grade 3 and 0 examinations as grade 4. Complete agreement between the 3 observers was present in 55.3% of all examinations. Scores of 2 of 3 reviewers agreed and that of the third reviewer differed by ≥ 1 grade in 42.9% of examinations. All three observers disagreed in 2.3% (n = 23) of observations. All observers agreed or 2 of 3 agreed and the third observer differed by ≤ 1 grade in 98.3% of examinations.

Inter-observer reliability for ACM was moderate (κ_w = 0.43, [95% CI: 0.38 to 0.48]; κ_w = 0.46 [95% CI: 0.41 to 0.51]); and κ_w = 0.56 [95% CI: 0.51 to 0.61]). Agreement between the 3 observers was observed for 952 examinations as grade 1, 1 examination as grade 2, 1 examination as grade 3, and 2 examinations as grade 4. Complete agreement between the 3 observers was observed for 95.5% of examinations. Scores of 2 of 3 observers agreed and that of the third observer differed by ≥ 1 grade in 4.5% of examinations. All

three observers disagreed in 0.2% (n = 2) of observations. All observers agreed or 2 of 3 agreed and the third observer differed by ≤ 1 grade in 100% of examinations.

Inter-observer reliability for TM was moderate (κ_w = 0.43, [95% CI: 0.39 to 0.47]; κ_w = 0.46 [95% CI: 0.42 to 0.50]; and κ_w = 0.57 [95% CI: 0.53 to 0.62]). Agreement between the 3 observers was observed for 187 examinations as grade 1, 66 examinations as grade 2, 70 examinations as grade 3, 12 examinations as grade 4 and 1 examination as grade 5. Complete agreement between the 3 observers was observed for 34.2% of examinations. Scores of 2 of 3 observers agreed and that of the third observer differed by ≥ 1 grade in 57.5% of examinations. All three observers disagreed in 9.2% (n = 90) of observations. All three observers agreed or 2 of 3 agreed and the third observer differed by ≤ 1 grade in 91.7% of examinations.

4.5 DISCUSSION

Tracheobronchoscopy offers the ability to quickly and accurately assess the upper and lower respiratory tract for EIPH, PLH, ACM and TM. Although previous investigators have utilized this technique, only one study reported on interobserver variability for assessment of the presence and severity of EIPH.[6] Interobserver variability may be affected by poor agreement between observers or lack of consistency within an individual observer. A highly reproducible and repeatable grading system would have great clinical and research applications. This would allow for more precise determination of the condition and be able to more accurately evaluate response to treatment.

Interobserver agreement was highest for classification of EIPH ($\kappa_w > 0.77$) as compared to a previous report that used a similar EIPH grade scale ($\kappa_w > 0.74$).[6] Lower interobserver agreements were seen with the PLH ($\kappa_w > 0.42$), ACM ($\kappa_w > 0.42$) and TM ($\kappa_w > 0.42$) grade scales. The magnitude of kappa is influenced by the extent of the agreement as well as by the prevalence of the condition. When the prevalence is very high or very low (outside the range of 0.2 to 0.8), the κ statistic becomes unstable and is difficult to interpret .[4] Since the prevalence of EIPH, PLH, ACM and TM was high in this study, especially at low grades, the κ statistic needs to be interpreted with this in mind. In addition the weighting matrix used will influence the final kappa statistic and this has not always been clearly specified in previous publications, which may explain slight differences between papers.

The observed proportion (OP) of agreement between 2 or more observers (that is the proportion of observations that the observers agree upon) in this study, differed by 1 grade or less in > 99%, > 98%, 100% and > 91% of examinations for EIPH, PLH, ACM and TM respectively, indicating good concordance using these grading systems. Despite the OP of agreement, between 2 or more observers being high for PLH, ACM and TM, weighted kappa was moderate and this may have been due to the high prevalence of the conditions and an over-representation of categories within each grade scale.

To fully evaluate association with performance, potential risk factors, and therapeutic interventions for EIPH, PLH, ACM and TM, grading systems which are reliable and repeatable are required. Quantification of EIPH has occurred in the past by

tracheobronchoscopic assessment and grading,[11] although the severity of EIPH may not be reflected by the grade allocation. Also, although no association has been proven, red cell counts in bronchoalveolar lavage fluid have been used to assess EIPH severity.[10]

Tracheobronchoscopy is a quick, safe, minimally invasive technique that may be performed on unsedated racehorses. It is a practical screening technique that may have prognosticative validity and clinical dependability and that would allow assessment of upper and lower respiratory tract of a large number of racehorses in field conditions. In this study, despite two of 3 reviewers being less experienced, excellent interobserver reliability was seen using the EIPH grading system[6] similar to a previous report that used three experienced observers.[6] Although the weighted kappa was lower for PLH, ACM and TM, this study demonstrated sufficient reliability to allow the use of the EIPH, PLH, ACM and TM grading system by veterinarians with limited experience and still achieve satisfactory clinical assessments.

4.6 CONCLUSIONS

Endoscopic grading of respiratory tract disorders is a relatively quick procedure which is easy to perform and eliminates the use of expensive time-consuming laboratory diagnostics. Moreover, it is a relatively safe diagnostic technique for both staff and racehorse. Using previously established grading criteria,[1,3,6,12] we demonstrated their reliability in the classification of EIPH, PLH, ACM and TM in racehorses competing in South Africa.

4.7 FIGURES AND TABLES

Figure 4.1 The portable flexible videoendoscopy system used in the grading of respiratory tract disorders in South African Thoroughbred racehorses.

4.8 REFERENCES

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Chapter 5 Proinflammatory mRNA response in racehorses with exercise-induced pulmonary haemorrhage

5.1 ABSTRACT

Exercise-induced pulmonary haemorrhage (EIPH) affects racehorses worldwide and may be due to stress failure of pulmonary capillaries. EIPH causes pulmonary neutrophilia and may be similar to acute lung injury in humans where neutrophil-mediated injury causes proinflammatory cytokine production. In an attempt to better understand the immunopathogenesis of EIPH, we designed a prospective, cross-sectional study of pre-enrolled Thoroughbred racehorses competing in flat races at high altitude (> 1,400 meters above sea level) and at sea level in a racing jurisdiction that does not permit the use of furosemide nor nasal dilator strips. After tracheobronchoscopy was performed < 2 hours after racing, the presence and severity of EIPH was graded 0 to 4 and venous blood was collected from 10 horses in each grade classification. Following RNA isolation and cDNA synthesis, real-time PCR was used to detect equine cytokine-specific mRNA for interleukin (IL) -1, -6, -10, interferon (INF) -γ, and tumor necrosis factor (TNF) -α. Overall, there was significantly greater expression of IL-6 and -10 within the different grades of EIPH ($P < 0.05$). Racehorses with a grade 4 versus 0, 1 and 2 EIPH expressed

increased IL-6 mRNA ($P < 0.05$), while greater IL-10 mRNA expression was present in horses with grade 3 versus 0 and 2 EIPH ($P < 0.05$). Overall, there was greater expression of IL-6 mRNA at sea level ($P = 0.009$) and TNF-α mRNA at altitude ($P = 0.005$). Although, it is unclear whether the inflammatory response observed in the study was due to pre-existing pulmonary inflammation or as a direct consequence of pulmonary bleeding, this study demonstrates a systemic correlation to pulmonary inflammation.

5.2 INTRODUCTION

Exercise-induced pulmonary hemorrhage (EIPH) is a worldwide phenomenon in Thoroughbred racehorses undergoing strenuous exercise with a reported prevalence of 43% to 75.4%.[38,39,42] No precise mechanism has been identified that can account for the site of occurrence and progression of EIPH within the lung; however pulmonary hypertension with secondary stress failure of pulmonary capillaries has been implicated.[48] EIPH is definitively diagnosed by post-exercise endoscopic examination of the upper respiratory tract and detection of blood in the trachea. Tracheal aspirates may reveal red cells and haemosiderophages while bronchoalveolar lavage may be used to quantify EIPH by measurement of red cell concentration.

Pulmonary inflammation is often seen in racehorses with EIPH.[33] This may be due to either pre-existing small airway disease[31] or due to the presence of blood in the airways.[30] Inflammatory chemical mediators may be intimately involved in airway inflammation.[40] We hypothesized that EIPH may be similar to acute lung injury in humans where

following post-traumatic haemorrhage, neutrophil-mediated injury may lead to local up-regulation of proinflammatory cytokines within the lungs. The parechymal lung inflammation may damage the alveolocapillary barrier leading to a systemic inflammatory response.

Because the determination of an association between EIPH and inflammation at a molecular level may assist in the development of preventative strategies aimed at reducing the prevalence and severity of this condition, we sought to measure interleukin (IL) -1, -6, -10, interferon (INF) -γ, and tumor necrosis factor (TNF) -α gene expression in a natural population of racehorses with EIPH immediately after racing in a racing jurisdiction that does not permit race day administration of furosemide nor nasal dilator strips, and in which horses race at both sea level and at high altitude (> 1,400 meters above sea level).

5.3 MATERIALS AND METHODS

5.3.1 Thoroughbred racehorses

The study was a cross-sectional study of a sample of Thoroughbred racehorses competing at Turffontein Race Course (Gauteng Province), Vaal Race Course (Free State Province), Clairwood and Greyville Turf Club (Kwazulu-Natal Province) and Kenilworth Race Course (Western Cape Province), in South Africa. Thoroughbred racehorses of either sex, running on turf or sand, competing in flat races were enrolled into the study between

August 4 and December 19, 2005. Race day administration of medications such as furosemide is not allowed in South Africa and drug testing is strictly enforced by the National Horse Racing Authority (NHRA) through screening of urine and blood for prohibited and therapeutic substances. Lists of available horses that were accepted to race were obtained from the NHRA. Eligible racehorses were then identified, trainers contacted individually and permission sought to examine the horse and draw blood. Only pre-enrolled horses (that is prior to race day) were entered into the study to avoid a potential enrollment bias.

5.3.2 Tracheobronchoscopy and sample collection

Tracheobronchoscopic evaluation was performed within 2 hours after racing on unsedated racehorses for evidence of EIPH using an endoscope (Pentax Corporation, Tokyo, Japan) that was passed through one of the nares, nasopharynx, larynx, to the level of the tracheal bifurcation. The severity of EIPH was immediately graded by one examiner according to a previously established grading system from 0 to 4[15] with grade 0 indicating the absence of blood in the pharynx, larynx, trachea, or mainstem bronchi; grade 1 indicating the presence of 1 or more flecks of blood or ≤ 2 short (< $^1/_4$ length of the trachea), narrow (< 10% of the tracheal surface area) streams of blood in the trachea or mainstem bronchi (Figure 2.1); grade 2 indicating a long stream of blood (> $^1/_2$ length of the trachea) or > 2 short streams covering < $^1/_3$ of the tracheal circumference (Figure 2.2); grade 3 indicating multiple, distinct streams of blood covering > $^1/_3$ of the tracheal circumference without blood pooling at the thoracic inlet (Figure 2.3); and grade 4

indicating multiple, coalescing streams of blood covering > 90% of the tracheal surface with blood pooling at the thoracic inlet (Figure 2.4).

Following allocation of a specific EIPH grade to each horse, 2.5 ml of venous blood was collected by routine jugular venipuncture from 10 horses in each EIPH grade classification (grade 0 to 4) directly into the Paxgene® RNA collection tubes (Qiagen, Valencia, CA) (Figure 5.1) within 2 hours after racing. Immediately following collection, the tubes were inverted 10 times to prevent coagulation that would hinder future extraction. The tubes were kept at room temperature overnight, and then stored at -20 °C until RNA extraction was performed.

5.3.3 RNA extraction and cDNA synthesis

Following thawing, cell pellets were isolated by centrifugation at 2,500 x *g* and RNA isolation carried out according to a modified manufacturer's protocol (Qiagen, Valencia, CA); after addition of Proteinase K, a 5 minute incubation period was added at room temperature before heating the samples to 55°C and the subsequent centrifugation was for 10 minutes at 16,000 x *g*. Total RNA was eluted in 40 ul RNase-free water (Figures 5.2 and 5.3) and then stored at -80 °C. Complementary DNA was synthesized according to the manufacturer's protocol (Qiagen, Valencia, CA).

5.3.4 Real-time polymerase chain reaction (real-time PCR)

Real-time PCR was performed on a 7500 Sequence Detection System machine (Applied Biosystems, Foster City, CA) (Figure 5.4). The five target genes of interest in this study were IL-1, -6, -10, TNF-α and IFN-γ. Applied Biosystems (Applied Biosystems, Foster City, CA) designed the primer and probe sequences for the cytokines and provided an Assay-by-Design (Applied Biosystems, Foster City, CA) kit containing both the designed primer and probe in solution (Table 5.1). In order to allow for potential variability in sample processing, the expression of the genes of interest were initially compared to β-glucuronidase (β-GUS). This control gene has been proven to have the lowest variability.[2] Additionally, relative quantiation (RQ) of gene expression was performed according to the method of Livak and Schmittgen[26] where the internal calibrator used was the average of grade 0 EIPH samples. Each cDNA sample was amplified in duplicate and all reaction solutions and samples were added to the plate using a robotic pipetting machine (EpMotion 5070, Eppendorf, Westbury, NY) (Figure 5.5 and 5.6) thereby allowing the study's samples to have the best pipetting accuracy and reproducibility. Also, a positive (LPS-stimulated lymphocytes) and a negative control (water) was included in each plate. The real-time PCR reaction mixtures had a final volume of 25 ul consisting of 10 ul of cDNA and 15 ul of the master mix. Amplification conditions were kept constant for all samples: 10 minutes at 95°C, 15 seconds at 95 °C, and 1 minute at 60 °C. The endpoint C_T was defined as the PCR cycle number that crosses signal threshold and ranged from 0 (no product) to 40.

5.3.5 *Data analysis*

Non-parametric tests were used to compare overall differences in target gene expression within the different grades of EIPH (Spearman's Rank-order correlation and Holm-Sidak t-test for multiple comparisons); and between location (altitude *versus* sea level) and EIPH grade (linear regression). Significance was set at $P < 0.05$. Statistical tests were conducted using commercially available computer software (SYSTAT®, Chicago, IL).

5.4 RESULTS

Mean expression of IL-1, -6, -10, INF- γ and TNF-α mRNA is depicted in Figures 5.7 to 5.11 respectively. While there was no statistically significant difference for mRNA expression of IL-1 ($P = 0.104$), TNF-α ($P = 0.06$), and INF- γ ($P = 0.36$) within the different grades of EIPH, significant difference was noted for IL-6 ($P = 0.046$) and IL-10 ($P = 0.02$) mRNA expression. Racehorses with a grade 4 EIPH expressed more IL-6 mRNA as compared to those horses with grade 0, 1 and 2 EIPH ($P < 0.05$), while racehorses with a grade 3 EIPH expressed more IL-10 mRNA compared to those horses with a grade 0 and 2 EIPH ($P < 0.05$). There was greater overall expression of IL-6 mRNA at sea level ($P = 0.009$), and TNF-α mRNA at altitude ($P = 0.005$). No significant difference was seen with the expression of IL-1 ($P = 0.82$), IL-10 ($P = 0.274$) and INF-γ mRNA ($P = 0.634$) between sea level and altitude.

5.5 DISCUSSION

Pulmonary inflammation in horses with more severe forms of EIPH is associated with histopathological evidence of small airway disease[33,34] and inflammation in bronchoalveolar lavage fluid and tracheal aspirates.[32] Whether the inflammation is a direct consequence of EIPH or if it predisposes to EIPH, is still not known. Autologous intrapulmonary blood inoculation in horses also causes prolonged airway inflammation.[30] Neutrophil-mediated injury may lead to intrapulmonary up-regulation of pro-inflammatory cytokines, damaging the alveolocapillary barrier causing a systemic inflammatory response.

In this study, we investigated mRNA IL-1, -6, -10, INF-γ, and TNF-α expression in a natural population of Thoroughbred racehorses with varying grades of EIPH competing at different altitudes. Although equine-specific monoclonal antibodies are not commercially available for IL-1, -6, -10, INF-γ, and TNF-α; and direct comparison can not be made between mRNA expression and protein levels; we assumed that mRNA expression reflected those of the biologically active cytokine. Furthermore, several studies have demonstrated a good correlation between inflammatory cytokine gene expression and disease conditions in the horse.[12,24,47]

We chose to study proinflammatory cytokines IL-1, -6 and TNF-α as these cytokines are responsible for induction of fever, neutrophil recruitment, tissue remodeling and immune activation[8] and INF-γ which is a pleotropic cytokine with proinflammatory properties that

augments TNF activity.[8] Interleukin-10 was studied for its potent anti-inflammatory activity as it may suppress proinflammatory cytokines such as IL-1 and TNF-α.

We have previously reported on the effect of altitude on the prevalence and severity of EIPH in Thoroughbred racehorses in South Africa using tracheobronchoscopy and concluded that EIPH is more prevalent ($P = 0.002$) and more severe ($P < 0.001$) at sea level.[42] EIPH may be assessed quickly and easily using tracheobronchoscopic examination, as this technique is minimally-invasive and allows immediate grading of racehorses with EIPH without laborious, time-consuming processing of samples in a laboratory. Although the repeatability of this tracheobronchoscopic grading system has been established[17] the relationship between the volume of blood in the airways and actual haemorrhage is not known. In this study, we assumed that horses with higher grades of EIPH were more severely affected and therefore suffered more haemorrhage.

Although pro-inflammatory responses have not been documented before in horses with EIPH, reports exist on increased mRNA expression of IL-1β, -8 and TNF-α in the bronchoalveolar lavage fluid of horses with recurrent airway obstruction,[14] increased mucosal IL-4 and -10 associated with the presence of Cyathostominae larvae in the equine large colon wall,[7] and increased IL-1β, -8 and TNF-α in blood leukocytes of horses following infection with *Anaplasma phagocytophilia*.[22]

Although a previous report found no significant effect of exercise on IL-4, -12 and IFN- γ mRNA expression,[3] the present study is, to the author's best knowledge, the first to report

an association between mRNA expression and EIPH. Racehorses with a higher grade EIPH and therefore more blood loss had greater pro-inflammatory IL-6 mRNA expression which was counter-regulated by a corresponding increase in anti-inflammatory IL-10 mRNA expression compared to lower grades of EIPH. Previous reports have also indicated that IL-6 expression may increase dramatically[19,44] with highest concentrations correlating with the volume of blood lost[31] and may remain elevated between 3[19] to 21[10] days. Expression of IL-6 can also increase in response to higher concentrations of TNF-α and IL-1, and is regarded as a pro-inflammatory cytokine which has anti-inflammatory properties.[4,35] Also, infiltrating neutrophils express after post-traumatic haemorrhage increased TNF-α mRNA in humans,[1] and there is up-regulation of this cytokine within 30 minutes after haemorrhage.[46] TNF-α is inhibited by IL-10 through stabilization of IκBα, preventing translocation of NF-κB.[25,49] In humans, IL-10 is the most important anti-inflammatory cytokine within the pulmonary innate immune response,[35] with anti-inflammatory properties[13,20,29] and is also up-regulated in the lung after haemorrhage[43] as was reported in this study.

In this study, altitude seemed to affect mRNA expression, as more IL-6 was expressed at sea level, while greater TNF-α expression was seen at altitude. Stressors (hypoxia, exercise) may initiate an immune and inflammatory response[27] characterized by increased IL-6 and TNF-α. In humans, exercise following acute exposure to high altitude was associated with increased IL-6 and not TNF-α expression,[15,23] while TNF-α is elevated after prolonged and intense exercise at sea level.[36] This study differs from previous reports[15,23,36] since IL-6 was increased at sea level and TNF-α greater at altitude. As

horses raced over shorter distances at sea level (as reported in Chapter 2), it is possible that overexertion over shorter race distance may have caused a more profound increase in IL-6 expression. Moreover, at altitude, racehorses competing over longer distances may have expressed more TNF-α as was found in human athletes.[36] Other plausible reasons exist for differences in cytokine expression and may include the use of fully-acclimatized horses that did not suffer hypoxaemia while racing at altitude (oxygen saturation was however not tested in this study), differences in actual elevation above sea level between the various studies, and that EIPH may elicit a different immune and inflammatory cytokine response.

Altitude and EIPH grade had no effect on venous IL-1 or INF-γ mRNA expression. In humans following trauma, IL-1 is undetectable within the first few hours[19] and can remain low for up to 5 days.[41] Interferon-gamma assists in immunomodulation, lymphocyte recruitment and activation and has anti-pathogen activity.[5] Through enhanced cell-mediated immunity, INF-γ causes a Type 1 response which results in destruction of virus-infected cells and recovery from infection. In the horse, production of INF-γ by $CD4^+$ and $CD8^+$ T cells in the lung of adult horses was associated with clearance of virulent *Rhodoccocus equi,*[18] equine infectious anaemia virus stimulated peripheral blood mononuclear cells to produce INF-γ,[11] infection with equine influenza virus or the use of a recombinant vaccinia Ankara viral vector resulted in increased expression of INF-γ mRNA,[6,45] and infection with equine herpes virus-1 resulted in age-related increased INF-γ production by peripheral blood mononuclear cells.[37] Since an infectious etiology has not been implicated in the pathogenesis of EIPH, it is not surprising that INF-γ which

affects cell-mediated cytotoxicity was consistently expressed at low levels in the racehorses irrespective of grade or location.

Although this study did not report the origin nor the cell type involved, it has been previously shown that intrapulmonary blood inoculation initially causes a local neutrophilic infiltration, followed by macrophages and to lesser degree lymphocytes.[30] Equine neutrophils have been demonstrated to produce proinflammatory IL-1, -6, -8, and TNF-α and not IL-4, -5, and INF-γ mRNA which is mainly produced by lymphocytes.[21] All together this suggests that following EIPH-induced pulmonary neutrophilia, the neutrophils may be actively involved in the observed systemic inflammatory response as reported in this study.

The mRNA expression of cytokine profiles in a natural population of racing Thoroughbreds presented in this report may assist in the understanding of the immunopathogenesis of EIPH. In future, gene linkage studies may prove useful in determining the susceptibility to EIPH by studying the balance of expression of proinflammatory and anti-inflammatory cytokines. Further research on therapeutic strategies which may include neutralizing antibodies, receptor antagonists, soluble receptors and inhibitors of proteases may be warranted.[9] This may interrupt the proinflammatory cytokine cascade and reduce the prevalence and severity of EIPH.

5.6 CONCLUSIONS

Results of this study indicate that increased IL-6, and -10 mRNA production is associated with more severe forms of EIPH. Also, there was greater expression of IL-6 mRNA at sea level and TNF-α mRNA at altitude. Although, it is unclear whether the inflammatory response observed in the study was due to pre-existing pulmonary inflammation or as a direct consequence of pulmonary bleeding, this study demonstrates a systemic correlation to pulmonary inflammation. Further studies are warranted to understand the relationship between cytokine expression and EIPH.

5.7 FIGURES AND TABLES

Figure 5.1 A PAXgene® Blood RNA Tube containing venous blood.

Figure 5.2 Pipetting the sample onto the PAXgene® RNA spin column during the RNA extraction procedure.

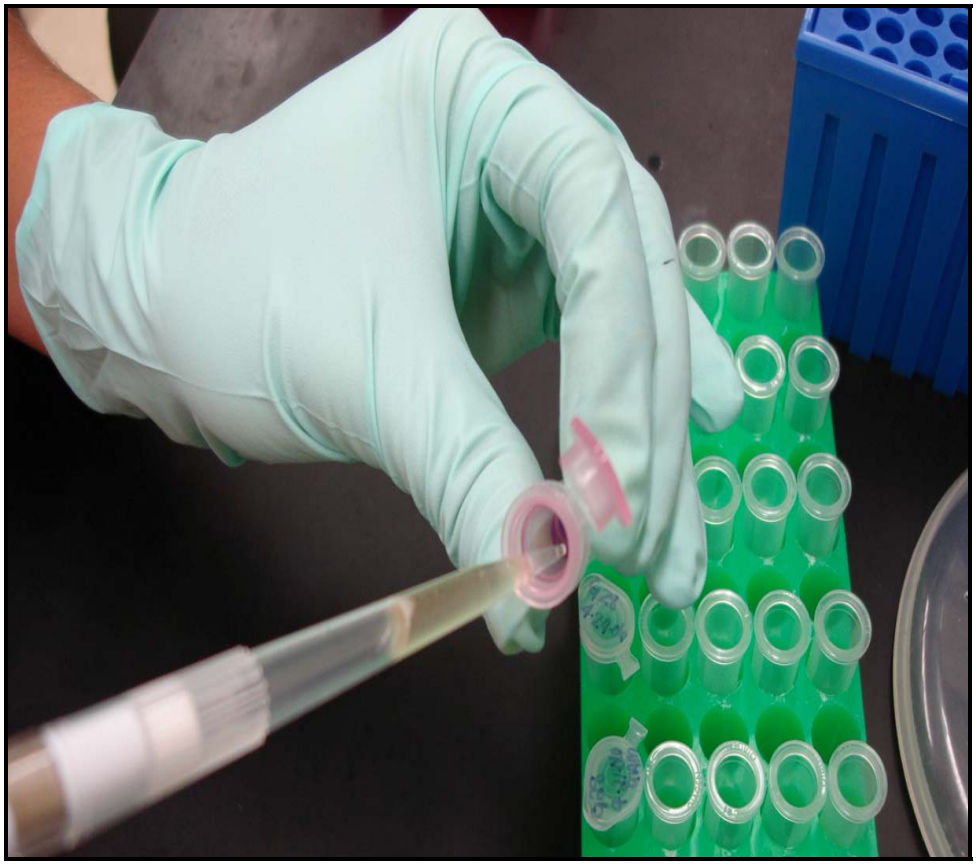


Figure 5.3 Preparing to perform RNA elution following centrifugation of the PAXgene® RNA spin column.

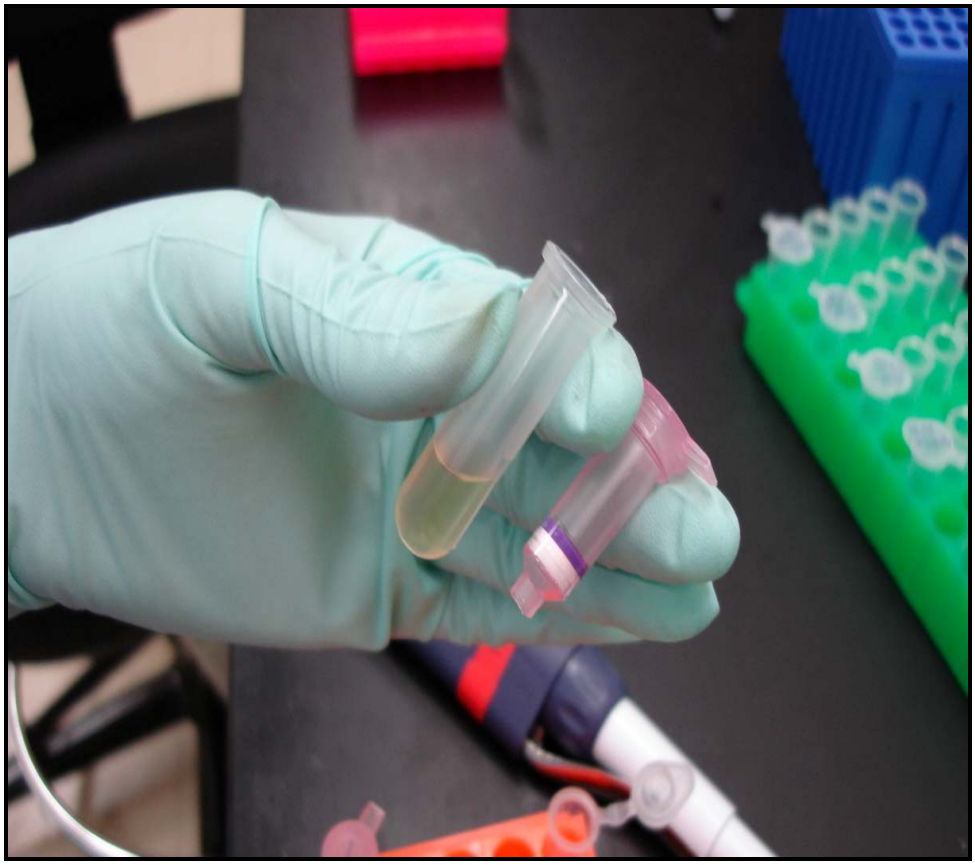


Figure 5.4 Preparing to perform real-time polymerase chain detection on the Applied Biosystems 7500 sequence detection system machine.

Figure 5.5 The epMotion 5070 robotic pipetting machine.

Figure 5.6 Primers and probes ready to be added to each cDNA sample by the epMotion 5070 robotic pipetting machine.

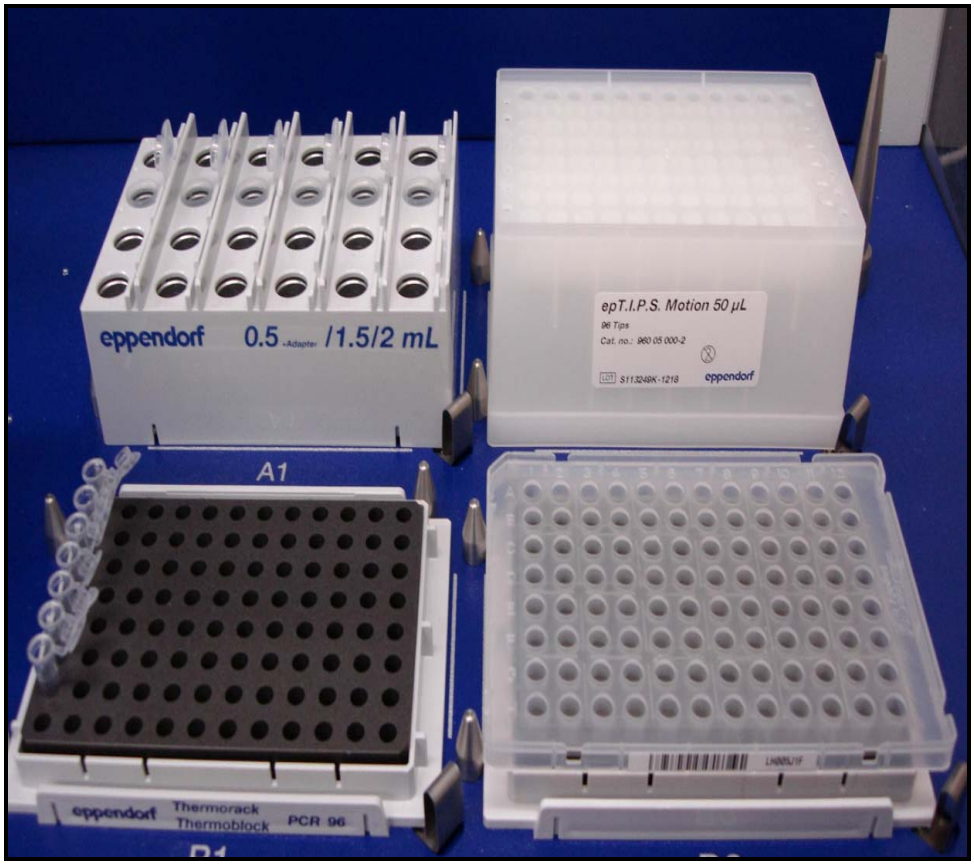
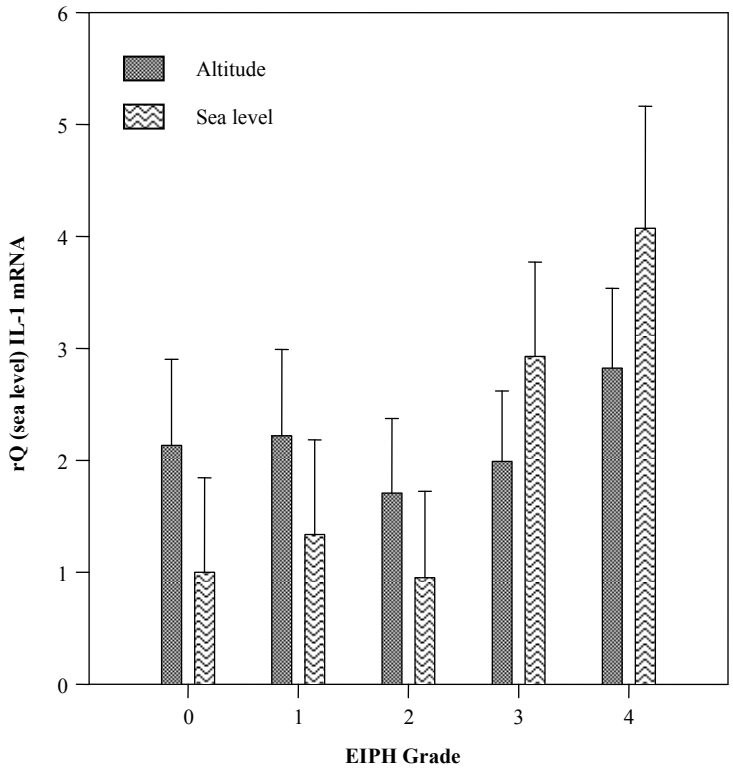
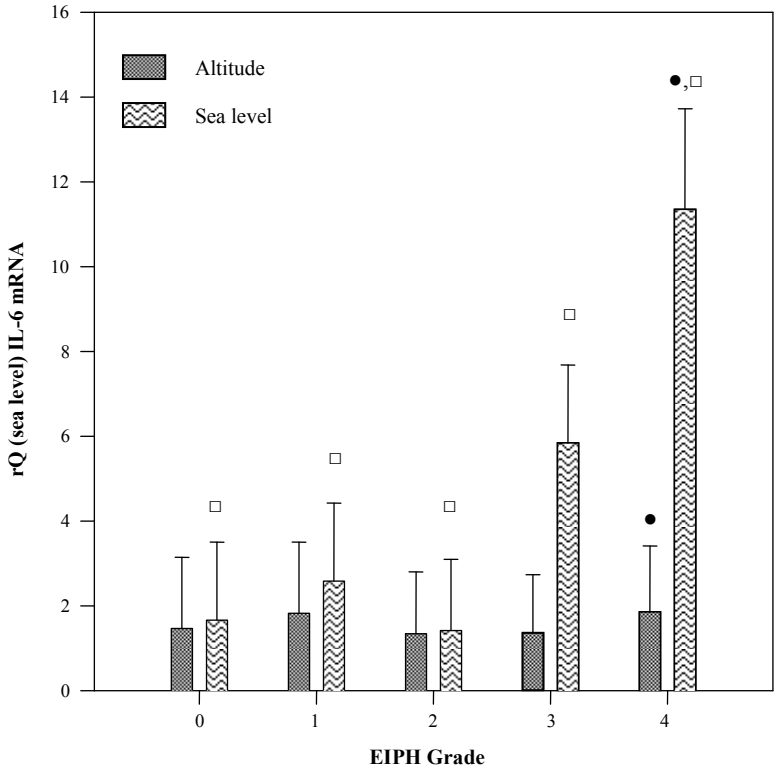


Figure 5.7 Expression of IL-1 mRNA in Thoroughbred racehorses with grade 0 to 4 exercise-induced pulmonary haemorrhage after racing at high altitude and at sea level.



No significant statistical differences existed between IL-1 mRNA expression and EIPH grade or location.

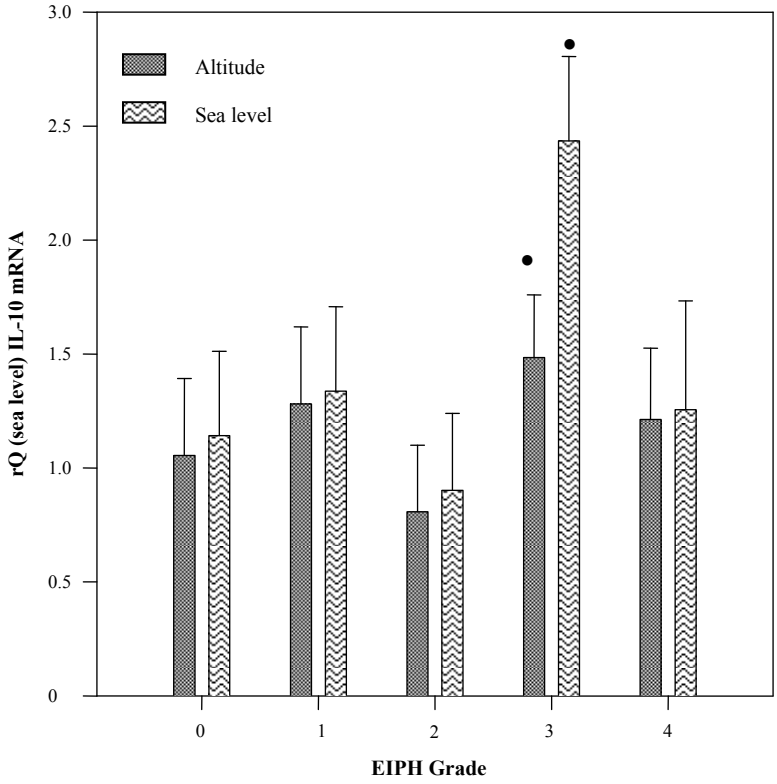
Figure 5.8 Expression of IL-6 mRNA in Thoroughbred racehorses with grade 0 to 4 exercise-induced pulmonary haemorrhage after racing at high altitude and at sea level.



• Significant differences ($P < 0.05$) existed between expression of IL-6 mRNA in racehorses with grade 4 *vs*. 0, 1 and 2 EIPH.

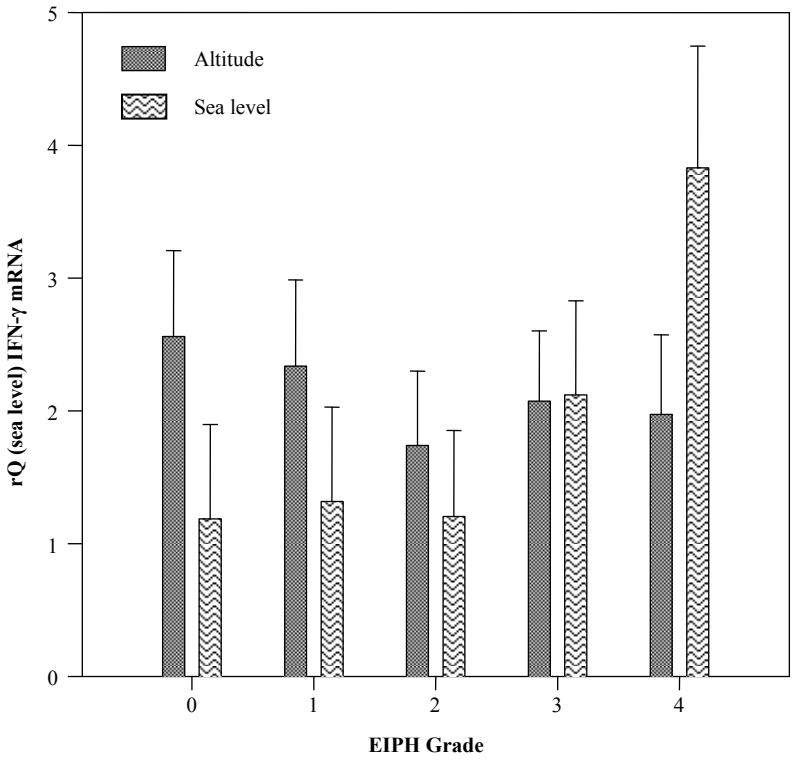
□ Significant differences ($P < 0.05$) in expression of IL-6 mRNA in racehorses at sea level compared to altitude.

Figure 5.9 Expression of IL-10 mRNA in Thoroughbred racehorses with grade 0 to 4 exercise-induced pulmonary haemorrhage after racing at high altitude and at sea level.



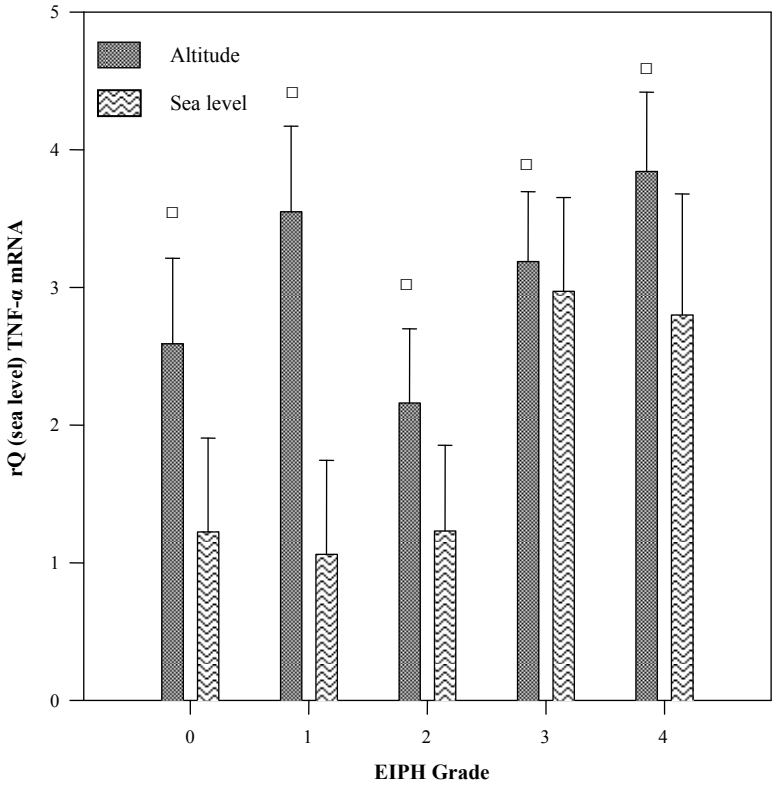
• Significant differences ($P < 0.05$) existed between expression of IL-10 mRNA in racehorses with grade 3 *vs*. 0 and 2 EIPH.
No significant differences existed between expression of IL-10 mRNA in racehorses and location.

Figure 5.10 Expression of IFN-γ mRNA in Thoroughbred racehorses with grade 0 to 4 exercise-induced pulmonary haemorrhage after racing at high altitude and at sea level.



No significant statistical differences existed between IL-1 mRNA expression and EIPH grade or location.

Figure 5.11 Expression of TNF-α mRNA in Thoroughbred racehorses with grade 0 to 4 exercise-induced pulmonary haemorrhage after racing at high altitude and at sea level.



□ Significant differences ($P < 0.05$) existed between the expression of TNF-α mRNA at altitude *vs*. sea level.

Table 5.1 Accession name and order number of target gene studied.

Gene	GeneBank Number	ABI Order Name
IL-1	U92480	EQIL-1B-JN2
IFN-γ	U04050	EQIFNGIS-JN3
IL-6	U64794	EQIL-6
IL-10	U38200	EQIL-10IS-JN2
TNF-α	M64087	EQTNFAIS-JN2
β-GUS	Not available	GUS

IL: interleukin
IFN: interferon
TNF: tumor necrosis factor
GUS: glucuronidase

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