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Effect of hospitalization on equine local intestinal immunoglobulin A (IgA) concentration measured in feces

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ABSTRACT

During hospitalization horses may develop gastrointestinal conditions triggered by a stress-associated weak local immune system. The prospective, clinical trial was conducted to find out whether fecal immunoglobulin A (IgA) concentrations could be determined in hospitalized horses and how they changed during hospitalization and in response to various stressors. Samples were obtained from 110 horses and a control group ($n = 14$). At arrival in the hospital, horses were categorized into pain grades (1-5), and elective versus strenuous surgery (> 2 hours, traumatic and emergency procedures). Feces were collected on day 1, day 2, day 3, and day 7 in all horses. Blood samples were obtained at the same intervals, but additionally after general anaesthesia in horses undergoing surgery (day 2). IgA concentration in feces was determined by ELISA and measured in optical density at 450nm. The control group showed constant IgA concentrations on all days (mean value 0.30 OD_{450} ±SD 0.11, 1.26 mg/g; $n = 11$). After general anaesthesia fecal IgA concentrations decreased considerably independent of duration and type of surgery ($P < 0.001$ for elective and $P = 0.043$ for traumatic surgeries). High plasma cortisol concentrations were weakly correlated with low fecal IgA on the day after surgery ($P = 0.012$, day 3, correlation coefficient $r = 0.113$). Equine fecal IgA concentrations showed a decline associated with transport, surgery, and hospitalization in general, indicating that stress has an impact on the local intestinal immune function and may predispose horses for developing gastrointestinal diseases such as enterocolitis.

1. Introduction

Immunoglobulin A (IgA) is a very important secretory component of the local immune system in horses and other mammals [1-3]. Secretory IgA has been identified as the predominant immunoglobulin in equine nasal secretions [4-6], saliva [6,7], and feces [8] and has been detected in intraocular samples [9,10].

In the gastrointestinal tract, it is the most secreted immunoglobulin in most species and plays an important role in the mucosal immune defence [1,11]. A decrease of IgA has been shown in various species to correlate with gastrointestinal diseases such as inflammatory bowel disease [12,13]. Gastrointestinal conditions like enterocolitis are common in hospitalized horses and are often associated with stress [14]. The effects of stressful events on mucosal immune responses have been widely analysed during inflammatory conditions in different species. Especially salivary IgA has been extensively studied in this context with contradictory results as there have been increases, decreases, or no changes in different studies [15-19]. Variable results have also been reported in fecal IgA concentrations [20-24]. Stressful situations like exercise, transport, hospitalization, surgery, and pain can regulate the mucosal immune system through two ways [25-28]. Firstly, through the release of catecholamines by the sympathetic-adrenal-medullary axis and secondly, through activation of the hypothalamic-pituitary-adrenal axis (HPA axis) [29-31]. Amongst other functions, glucocorticoids are known to have strong anti-inflammatory and immunosuppressive effects but also enhance adaptive immunity and therefore the production of immune cells such as plasma cells in the mucosa. In this context, the intensity of the stressor modulates the production of secretory IgA [29, 30]. Studies have shown that secretory IgA increases under acute stressful conditions but decreases with chronic stress in rats, mice, and pigs [32-35].

In this study, fecal IgA was assessed in hospitalized horses and a healthy control group as the effects of stress on the equine intestinal secretory immune system have not been explored in detail. Furthermore, pain grades and serum cortisol concentrations as established stress parameters were correlated with fecal IgA. Moreover, the usability of non-

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invasive fecal collection in these horses was evaluated.

2. Materials and Methods

2.1. Ethical approval

The study was approved by the Bavarian Government Animal Ethics Committee (No. 55.2-1-54-2531.2-25/07). Data were collected between March and November 2006 in the equine hospital of Ludwig-Maximilians-University in Munich, Germany.

2.2. Hospitalized horses

110 adult horses (mean age 8.9 years, ±SD 5.9) of different breeds which were hospitalized during the sampling period were included in the study. In addition to mainly Warmblood horses (83), the breeds consisted of Arabians (7), Thoroughbreds (4), Ponies (12) and draft horses (4) and any gender was included. There was no special selection of diseases, so that the normal case load treated in an equine hospital was represented. The diseases comprised surgical and internal medicine cases, various organ systems were affected (gastrointestinal, musculoskeletal, neurologic, ophthalmologic, endocrinologic, metabolic, urinary conditions) and elective as well as emergency cases were admitted.

After thorough clinical examination the horses were categorized into two groups: Horses undergoing surgery and horses exclusively receiving medical treatment. On arrival in the hospital the horses were further categorized into different pain grades, ranging from 1 to 5 (Table 1). Fecal IgA concentrations were measured in the morning (9 am) on day 1, day 2, day 3, and day 7. Serum cortisol concentrations were measured on day 1, day 2 (before (9 am) and after surgery), day 3 and day 7.

2.3. Control group

To compare the equine patients' fecal IgA concentrations and serum cortisol levels with healthy horses, a group of 14 healthy Warmblood horses (3 mares, 8 geldings, 3 stallions) with a mean age of 11.4 years (±SD 4.6) were sampled in their home stable environment. The animals had not been transported, subjected to any medical or surgical treatment, or stressed otherwise in the preceding six weeks. Fecal IgA and serum cortisol concentrations were assessed in the morning (9 am) at the same intervals (day 1, day 2, day 3, day 7) as in hospitalized horses.

2.4. Study design

After arrival in the hospital, feces and blood samples were collected (day 1). Only horses undergoing elective surgery on day 2 were included in the study, so the same pattern could be used as for the horses that did not undergo surgery. In the surgical patients, blood samples were taken on day 1, before and after surgery on day 2, on day 3 and on day 7. The sampling scheme varied for horses with emergency surgeries (e.g., colic horses) as they had surgery directly after arrival (first and second blood sample before and after surgery, optional fecal sample) and were sampled a third time on the next day. Horses receiving medical treatment were also sampled four times: On day 1, day 2, day 3, and on day 7. The same pattern was used for the control group. Some horses, especially colic patients, did not defecate on all days needed. Those horses were excluded from the study as it was decided against taking samples from the rectum to not additionally stress the horses.

2.5. Effect of freezing Samples and different Sampling Locations on IgA Concentration in Feces (preliminary work)

To determine the effect of freezing and sampling location on IgA concentrations in feces, ELISA was performed on samples that had been frozen for different time periods (no freezing, freezing for 1, 2, 3 days, 1 week and 1 month). Moreover, samples were taken from different sites of feces of the same day (fresh versus old fecal piles, middle and periphery left and right). There was no difference between concentrations at different sampling locations of feces of the same day (Mean (SD) in OD_{450}: horse 1: 0.28 (0.01), horse 2: 0.30 (0.01), horse 3: 0.47 (0.01), horse 4: 0.33 (0.02), respectively). Freezing samples at -20°C did not have an influence on IgA concentrations. Nevertheless, for this study fresh fecal samples from the middle of the droppings were frozen and evaluated within one week.

2.6. Feces sampling and processing

20 g feces were collected from the middle of fresh droppings and put into tubes (Feces tube, Sarstedt, Germany). All samples were immediately frozen (-20°C) and analysed within one week of sampling.

2.7. Analysis of feces

IgA concentrations were determined using a modification of the method described by Hau et al. [38].

Feces samples were defrosted at room temperature and manually

Table 1
Pain grades 1-5 (none to very severe) which were used to categorize the horses in this study. Table modified from Gleerup et al. 2016 [36,37].

Behaviour Pain	1 None	2 Mild	3 Moderate	4 Severe	5 Very severe
Grimace Scale/Pain face	None	Mild pain face present	Pain face present	Intense pain face	Intense pain face
Visible pain behaviour*	None	Occasional	Occasional	Continuous	Continuous
Activity	Interested, attention towards surroundings	No movement	No movement or starting to become restless	Restless	Raging or depressed
Head position	Eating, below withers or high	Level of withers	Below withers	Below withers or constantly changing	Constantly changing
Attention towards painful region	No attention to painful area	No or brief attention to painful area	Brief attention to painful area (e.g., flank watching)	Looking at painful area, biting	Throwing painful area against walls (severe colic)
Interactive behaviour	Interacts with observer (looking, moving towards)	Looks at observer, does not move	Does not look at observer, moves away, avoids contact	Not moving, not reacting/introverted	Not responding to environment
Response to food	Takes food without hesitation	Looks at food	Looks at or no response to food	No response to food	Not responding to environment
Vital parameters	Heart rate 28-40 bpm Respiratory rate 8-16 bpm	Heart rate 48-52 bpm Respiratory rate 20-28 bpm	Heart rate 54-60 bpm Respiratory rate 20-40 bpm	Heart rate 54-80 bpm Respiratory rate may be elevated	Heart rate > 80 Respiratory rate elevated

* Pain behaviour includes all visible behaviours like excessive head movements, flehmen, kicking, pawing, rolling, tail swishing, mouth playing, repeated stretching etc.

mixed. Then 9 g of the feces were dissolved in 18 ml PBS-buffer (pH 7.2). The samples were stirred again for 3 minutes and afterwards centrifuged for 10 minutes at 4000 rpm (Centrifuge 5610 R, Eppendorf, Germany). The supernatant fluid was removed, filled into small tubes (Microtube 1.5 mL, Sarstedt, Germany) and again centrifuged (10 min, 13200 rpm, Centrifuge 5415 R, Eppendorf, Germany). Homogenates were immediately analyzed for IgA.

A 96-well flat bottom polystyrene microtiter ELISA plate (Screen Mates, Matrix Technologies Corp.) was coated with 100 µL of the feces samples in 1:2 dilution with 100 µL layering buffer per well. The samples were layered to duplicate wells and the ELISA was replicated over a second plate run in parallel. No-sample controls were included on every plate. Coated plates were incubated overnight at 4°C and washed 3 times with phosphate buffered saline solution (PBS) containing 0.05 % Tween 20 (PBS-T, 200 µL per well). The uncoated sites of the wells were blocked by addition of 100 µL of blocking solution (1 % bovine serum albumin in PBS-T) to each well for 1 hour at 37°C. After decanting the blocking solution and washing the plates 3 times with PBS-T, 100 µL of the antibody (goat anti-horse IgA:HRP 1 mg/mL, Serotec, Duesseldorf, Germany) were added to duplicate wells. The antibody was used in a 1:10000 dilution in PBS-T. Following the addition of the antibody, the plates were incubated for 1 hour at 37°C and washed 3 times with PBS-T. After a final wash 100 µL of substrate consisting of 332 µL of tetramethylbenzidine (TMB), 10 ml TMB-buffer and 3.3 mL 30 % hydrogen peroxide was added to each well. The reaction was stopped after 10 minutes by addition of 50 µL of H_2SO_4 to each well. Absorbance at 450 nm was read in an automated microplate reader (Sunrise, Tecan, Switzerland). IgA was measured in OD_{450} (optical density measured at 450 nm) and duplicate results averaged. To assess inter- and intra-assay variability controls were placed on the plates and reproducibility tested with duplicate plates. Overall assay variability was <12 %. A standard curve was generated by dilution of the antibody with known concentration to accurately determine the concentration of fecal IgA in mg/g dry matter (DM). To account for variations in fecal moisture, 1 g of each sample was oven-dried for 24 hours at 65°C to evaluate dry weight. Fecal concentrations of IgA were calculated based on each samples' dry matter (DM) content.

2.8. Blood sampling and processing

Blood samples were taken from the jugular vein in most horses or other available veins if the jugular veins were not accessible (EDTA and serum blood tubes, Sarstedt, Germany). To not additionally stress the horses by this procedure no restraint techniques were used apart from a halter. The blood samples were centrifuged, and serum was obtained. Serum samples were analysed for cortisol using an "Elecsys 1010" (Roche Diagnostics GmbH, Mannheim, Germany).

2.9. Statistical Analysis

Statistics were performed using SPSS (Windows version 11.5). The Shapiro-Wilk test was used to determine if results were normally distributed. For comparison of means and calculation of significance T tests were used for normally distributed data. For paired and independent random samples Mann Whitney tests, Kruskal Wallis tests, Levene tests or Wilcoxon tests were used. Spearmańs correlation calculations were used to assess correlation of IgA levels and serum cortisol levels. Results for each group (healthy, non-surgery and surgery group were expressed as mean ±SD (standard deviation). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Fecal IgA concentrations in healthy control horses

The variation of intra- and interindividual IgA concentrations was minimal in horses of the control group on all four days (mean value 0.30 OD_{450} ±SD 0.11 ≙ 1.26 mg/g; $n = 11$).

3.2. IgA in hospitalized horses

3.2.1. IgA concentrations after transport, general anaesthesia, and surgery

After transport to the equine hospital IgA concentrations of the healthy hospitalized horses (e.g., castrations) was equivalent to the control horses (0.33 OD_{450} ±SD 0.97 ≙ 1.26 mg/g; $n = 18$ and 0.31 OD_{450} ±SD 0.08 ≙ 1.24 mg/g; $n = 11$ on day 1, respectively).

During the first two days of hospitalization, IgA concentrations significantly increased in the group of elective surgeries (0.53 OD_{450} ±SD 0.14 ≙ 2.15 mg/g on day 2; $n = 12$; T-Test; $P = 0.008$). On the day after elective surgery, horses displayed lower IgA concentrations than before (0.19 OD_{450} ±SD 0.09 ≙ 0.70 mg/g; $n = 34$ on day 3, $P < 0.001$). On the fifth day after surgery concentrations had increased (0.44 OD_{450} ±SD 0.28 ≙ 1.78 mg/g; $n = 30$, day 7).

IgA concentrations significantly decreased after general anaesthesia and surgery of more than 2 hours duration independently of surgical trauma. After prolonged anaesthesia (> 2 hours) and traumatic surgery (e.g., colic surgery) mean concentrations were 0.18 OD_{450} ≙ 0.73 mg/g (±SD 0.15, $n = 7$ on day 3, $P = 0.043$) and did therefore not differ significantly from minor interventions.

Two horses (no. 40 and 78) were subjected to general anaesthesia for magnetic resonance imaging (MRI) only. These two horses showed decreased IgA concentrations after general anaesthesia without surgery (Table 2).

3.2.2. Pain grades and association with IgA concentrations

IgA concentrations of the most seriously ill and painful horses (e.g., colic horses, fractures) showed a tendency to stay lower than concentrations of the horses that were less severely ill and less painful. As horses categorized in pain grade 5 were mainly colic patients and surgery had to be performed immediately after arrival, no feces prior to surgery could be taken in some horses.

Pain grades correlated with IgA concentrations in horses after arrival at the equine hospital (day 1). Horses categorized in grade 4 (severe pain signs) displayed mean IgA concentrations of 0.09 OD_{450} (±SD 0.05, ≙ 0.25 mg/g; $n = 3$), whereas horses categorized in grade 3 had mean concentrations of 0.14 OD_{450} (±SD 0.14, ≙ 0.35 mg/g; $n = 7$) and horses in grade 2 displayed mean concentrations of 0.38 OD_{450} (±SD 0.07, ≙ 0.75 mg/g; $n = 19$).

Horses categorized in grade 1 (no pain signs) showed mean concentrations of 0.27 OD_{450} (±SD 0.14, ≙ 1.24 mg/g; $n = 29$). The difference between grade 2 and grade 4 was statistically significant ($P = 0.040$) (Fig. 1).

3.2.3. IgA in horses with gastrointestinal diseases

Horses with gastrointestinal diseases showed fluctuating IgA concentrations. One horse with colon torsion (horse no. 59) had very high IgA concentrations at arrival at the hospital (2.60 OD_{450}, ≙ 30.43 mg/g, day 1), whereas another horse with colitis had the lowest measured concentrations before occurrence of the disease (horse no. 89, <0.05 OD_{450}, ≙ 0 mg/g, day 1). In the course of disease IgA concentrations

Table 2
IgA concentrations of two horses subjected to general anaesthesia (GA) without surgery (measured in OD_{450}). Both horses showed decreased IgA concentrations on the day after general anaesthesia corresponding to the decrease in horses that were also subjected to surgery.

Horse no.	IgA concentration before GA (in OD_{450}, day 2)	IgA concentration one day after GA (in OD_{450}, day 3)	IgA concentration on day 5 after GA (in OD_{450}, day 7)
40	0.46 (≙ 2.0 mg/g)	0.10 (≙ 0.25 mg/g)	0.32 (≙ 1.26 mg/g)
78	1.58 (≙ 10.25 mg/g)	0.08 (≙ 0.22 mg/g)	0.55 (≙ 2.20 mg/g)

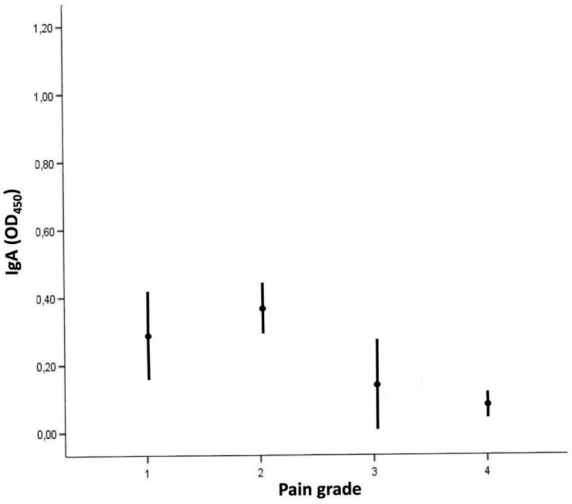

Fig. 1. Association between fecal Immunoglobulin A concentrations and pain grades (1=none to 4=severe) after arrival in the equine hospital (day 1). Horses categorized in grade 1 (no pain) showed mean concentrations of 0.27 OD_{450} (±SD 0.14, ≙ 1.24 mg/g; n = 29), whereas horses with grade 3 had mean concentrations of 0.14 OD_{450} (±SD 0.14, ≙ 0.35 mg/g; n = 7) and horses in grade 2 displayed mean concentrations of 0.38 OD_{450} (±SD 0.07, ≙ 0.75 mg/g; n = 19).
When individual groups were compared, only the difference between grade 2 (mild pain) and grade 4 (severe pain; 0.09 OD_{450} (±SD 0.05, ≙ 0.25 mg/g; n=3) was statistically significant (p=0.040).

increased up to 3.20 OD_{450} (≙ 33.2 mg/g, day 3) to decrease again on day 7 (0.11 OD_{450}, ≙ 0.27 mg/g). The same course of fecal IgA concentrations occurred in horse no. 69 with caecal tympany when it developed a peritonitis. IgA concentrations in this case increased from 0.14 OD_{450} (≙ 0.50 mg/g day 1) to 1.41 OD_{450} (≙ 7.32 mg/g, day 3). Horse no. 51 which was admitted with colitis had high concentrations at arrival (0.93 OD_{450}, ≙ 2.72 mg/g, day 1) which decreased to 0.12 OD_{450} (≙ 0.28 mg/g, day 3) and stayed on a very low level after laparotomy was performed (0.09 OD_{450}, ≙ 0.25 mg/g, day 7).

After surgery, fecal IgA concentrations in some horses with intestinal diseases increased. Horse no. 82 had IgA concentrations of 0.18 OD_{450} (≙ 0.73 mg/g) on day 1 which increased to 2.11 OD_{450} (≙ 23.80 mg/g) on the day after surgery (day 3). In other horses with gastrointestinal conditions IgA concentrations stayed constantly low (horse no. 33: mean IgA 0.09 OD_{450} (≙ 0.25 mg/g); horse no. 49: mean IgA 0.15 OD_{450} (≙ 0.55 mg/g)) (Table 3).

Table 3
Variations in the course of fecal IgA concentrations during hospitalization in horses with gastrointestinal diseases ($n = 7$).

Horse no.	Gastrointestinal condition	IgA day 1 (in OD_{450})	IgA day 3 (in OD_{450})	IgA day 7 (in OD_{450})
33	Inguinal hernia (incarcerated)	0.06 (≙ 0.20 mg/g)	0.10 (≙ 0.25 mg/g)	0.09 (≙ 0.25 mg/g)
49	Ileum impaction	0.09 (≙ 0.25 mg/g)	0.08 (≙ 0.22 mg/g)	0.27 (≙ 1.24 mg/g)
51	Colitis	0.93 (≙ 2.72 mg/g)	0.12 (≙ 0.28 mg/g)	0.09 (≙ 0.25 mg/g)
59	Colon torsion	2.60 (≙ 30.43 mg/g)	0.21 (≙ 0.75 mg/g)	0.36 (≙ 1.70 mg/g)
69	Caecal tympany	0.14 (≙ 0.50 mg/g)	1.41 (≙ 7.32 mg/g)	0.07 (≙ 0.20 mg/g)
82	Pedunculated lipoma	0.18 (≙ 0.73 mg/g)	2.11 (≙ 23.80 mg/g)	-
89	Colitis	<0.05 (≙ 0 mg/g)	3.20 (≙ 33.2 mg/g)	0.12 (≙ 0.28 mg/g)

3.3. Serum cortisol in hospitalized horses

Healthy horses brought to the hospital for elective surgeries (e.g., castrations) showed higher mean serum cortisol concentrations on day 1 than the healthy control group (Table 4), but the difference was not statistically significant ($P = 0.663$).

The influence of the different pain grades (1 to 5) on serum cortisol concentrations was significant ($P < 0.001$) indicating that categories were appropriate to assess pain in horses. Horses categorized in pain grade 1 ($n = 54$) had a mean serum cortisol concentration of 149.1 nmol/l (±SD 69.5), whereas horses categorized in pain grade 4 ($n = 9$) had a mean concentration of 367.9 nmol/l (±SD 112.8). Three horses with very severe pain (pain grade 5) had a mean concentration of 431.7 nmol/l (±SD 129.8) (Fig. 2).

Horses with mild diseases which were subjected to short general anaesthesia and minor surgical trauma ($n = 29$; e.g., ophthalmic surgeries) had elevated mean cortisol concentrations after transport to the hospital (161 nmol/l; day 1) and after surgery on day 2 (mean concentration of 214.9 nmol/l; ±SD 81.7). One day after surgery (day 3) mean serum cortisol concentrations decreased to lower than at admission (137.2 nmol/l, ±SD 61.8; $n = 45$, $P < 0.001$; Fig. 3).

During hospitalization and surgeries with major trauma (e.g., laparotomies, n=10) mean cortisol concentrations were higher than with minor surgeries, with mean concentrations of 264.1 nmol/l (±SD 154.4) before surgery and 329.8 nmol/l (±SD 82.2) after surgery (both day 2). This increase proved to be significant ($P < 0.001$). The day after surgery (day 3) cortisol decreased to lower concentrations within the reference range (mean concentration 149.7 nmol/l, ±SD 116.8, n=8; Fig. 4).

3.3.1. Association between serum cortisol and fecal IgA

To evaluate whether cortisol concentrations had an influence on fecal IgA concentrations, correlation of these parameters was assessed and found to be generally low. When assessed on the day after surgery (day 3), high serum cortisol concentrations were weakly correlated with low fecal IgA concentrations (Spearman ranking; $P = 0.012$; correlation coefficient $r = 0.113$; Fig. 5).

4. Discussion

This study aimed to investigate the course of fecal IgA concentrations during hospitalization in various horses subjected to different stressors, e.g., transport, new environment, anaesthesia, and different types of surgeries.

Secretory IgA is produced by a B-cell system which is part of the most important adaptive immune response mechanisms and serves as a first-line defence providing antibody functions on the mucosal surface [39-41]. The local mucosal immune system is the largest effector organ of humoral immunity and aims at preventing microbial colonization and penetrations of antigens through the epithelial barrier [3,42].

In most species, locally produced IgA exerts important functions in the gastrointestinal tract and on other mucosal surfaces, neutralizing lipopolysaccharides, and viruses, and suppressing attraction of neutrophils, eosinophils, and monocytes, and thereby reducing proinflammatory activities [2,43]. Furthermore, IgA can downregulate the

Table 4
Mean serum cortisol concentrations (and standard deviations) on day 1 in control horses and healthy horses that were transported to the hospital (e.g., for castration).

	Mean concentration	Standard deviation (±SD)
Serum cortisol concentration of control horses (n=14)	149.8 nmol/l	88.6
Serum cortisol concentration of healthy transported horses (n=33)	161 nmol/l	76.2

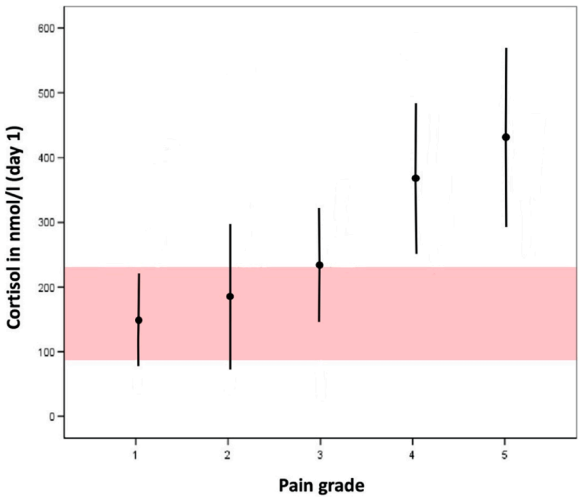

Fig. 2. Serum cortisol concentrations in association with the pain grades. Pain grades were categorized as follows: grade 1 = no pain (n=54), grade 2 = mild pain (n=23), grade 3 = moderate pain (n=21), grade 4 = severe pain (n=9), grade 5 = very severe pain (n=3). Pink coloured bar shows reference range in the laboratory performing the tests (reference range: 85-230 nmol/l).

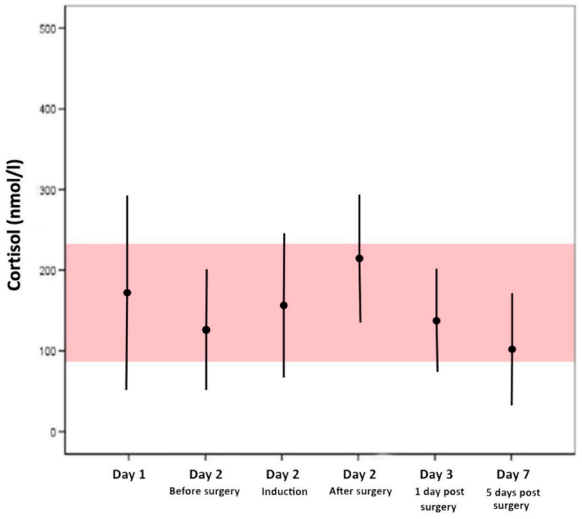

Fig. 3. Serum cortisol concentrations in horses subjected to short anaesthesia with mild surgical trauma (e.g., minor ophthalmic surgeries) during hospitalization. Pink coloured bar shows reference range in the laboratory performing the tests (reference range: 85-230 nmol/l).

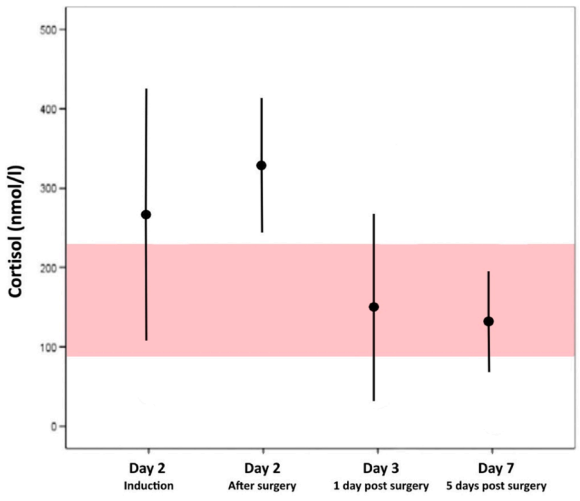

Fig. 4. Serum cortisol concentrations in horses which had to have long interventions (>2 hours) with major surgical trauma (e.g., laparotomies). As the horses were put under general anaesthesia shortly after admission, the first blood sample was taken when anaesthesia was inducted. The increase in serum cortisol concentration was highly significant ($p < 0.001$). Pink coloured bar shows reference range in the laboratory performing the tests (reference range: 85-230 nmol/l).

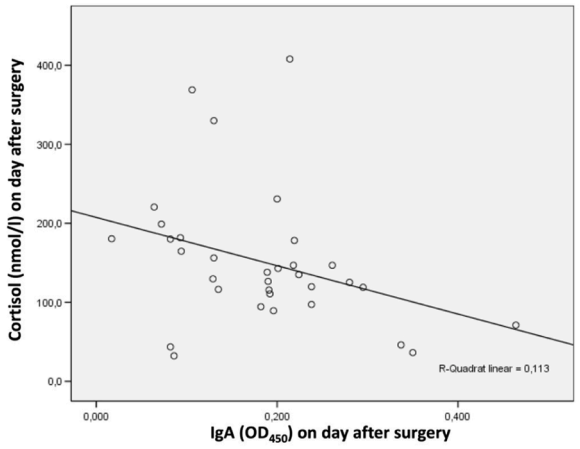

Fig. 5. Association between serum cortisol concentrations and fecal Immunoglobin A concentrations on the day after surgery (day 3). For this time point, high cortisol concentrations significantly correlated with low fecal IgA levels (p=0.012, r=0.113).

secretion of proinflammatory cytokines such as tumor necrosis factor alpha from activated monocytes [44] and inhibit neutrophil and monocyte activation that results in generation of reactive oxygen metabolites [45]. Overall, it supports in enhancing the nonspecific immune defenses and helps maintain balanced microbiota in the gut [46,47]. Vaerman et al. (1971) stated that horses, like other mammals, have an IgA system analogous to that of humans [1]. Furthermore, IgA is also known to be the immunoglobulin having the highest secretion and being produced by the majority of immunoglobulin-containing cells in the equine intestine. In humans, the IgA system is altered in several chronic respiratory diseases, such as chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis [48]. Serum IgA deficiency has been associated with higher exacerbation risk in COPD as well as with increased incidence of allergic diseases [49,50]. Pathogen-specific IgA can also be synthesized in response to infection [51-54]. In sheep and cattle, local production of IgA has been used to select for natural immunity to gastrointestinal nematodes as salivary assays show which animals express the highest levels of specific mucosal IgA [55,56].

Whereas evaluation of IgA in equine serum has been extensively studied, there is still lacking knowledge about local production of immunoglobulins in this species [8,57]. Some studies investigated local immune responses in the oral mucosa. It was found that there is significant production of IgA in the oral cavity in horses which can be detected in equine saliva [7]. Another study investigated the mucosal humoral immune response in horses after infective challenge with equine herpesvirus-1 and found high amounts of virus-specific IgA antibodies in nasal wash fluid [58]. Concentrations of IgA have also been determined in equine tears and were found to constitute 50 % of all lacrimal proteins [59].

Knowledge on local immunity in the equine intestinal tract is scarce. A study aiming on identifying noninvasive biomarkers for intestinal permeability found that there was no detectable local inflammation represented by a change in fecal IgA concentrations when horses were

given antibiotics or Lactobacillus cultures [60]. As fecal IgA significantly correlated with bacterial variations in the colon, an association between dysbiosis and the loss of mucosal integrity in equine large intestine was suggested [60]. Changes in the microbiota and dysbiosis was present in horses with colitis and laminitis, but the changes were considered to be only minor [61]. Nunn et al. (2007) found specific IgA antibodies to *Clostridium botulinum* especially in the jejunum and ileum in horses affected with grass sickness indicating a strong mucosal immune response [62].

According to earlier studies in rats, long-term stressors are capable of reducing IgA concentrations significantly [20,38], whereas short-term stressors did not show any influence on fecal IgA in earlier studies [21]. As maintained by the results obtained in the current study, horses showed decreased fecal IgA concentrations after stressors like transport and general anaesthesia. After arrival in the hospital horses displayed similar IgA concentrations as the control group, but IgA concentrations increased significantly following the first days in the equine clinic. This increase may be caused by contact with unknown antigens as the horses had to cope with an unfamiliar microbiome e.g., in the feed. Studies have proven that IgA is synthetized by mucosal plasma cells after local antigen stimulation [63-65]. Changes in the diet may alter the microbial community and their metabolites and affect the intestinal tissue [66,67].

Fecal IgA significantly decreased after surgical intervention from mean concentration of 0.53 OD_{450} (2.20 mg/g) prior to surgery to a mean concentration of 0.19 OD_{450} (0.70 mg/g) after elective surgery in horses. These results agree with earlier studies which show the impact on immune reactions after surgery [68-71]. On the contrary, medical procedures in mice (e.g., surgery involving the creation of a skin incision, abdominal flap, and subsequent suture) led to elevated salivary IgA concentrations that subsequently returned to baseline levels after 24h [72]. Surprisingly, horses undergoing very invasive surgeries in this study did not experience more distinct decreases of fecal IgA levels compared with the horses minor surgeries were performed on. Two horses, which were put under general anaesthesia for MRI but did not undergo surgery also showed very low IgA concentrations after general anaesthesia. This may indicate that stress associated with general anaesthesia and the following recovery period has a great impact on immune functions in the equine intestine as the pathogenesis of gastrointestinal disease should be viewed in the light of the obtained knowledge on the immunophysiology of gut mucosa and the gut-associated lymphoid tissue (GALT) [30]. To evaluate the impact of stress on the horses and to interpret the concentrations of fecal IgA, serum cortisol as an established stress parameter was measured accordingly. Serum cortisol was shown to increase after transport, general anaesthesia, and surgery in the horses undergoing various procedures. But only the high cortisol concentrations on the day after surgery were associated with low IgA concentrations in equine feces in the current study. These findings are similar to the results obtained in a study where higher cortisol concentrations in children were associated with lower secretory IgA concentrations and a higher incidence of illness [73]. Cortisol has a strong impact on the systemic immune system, and it has been shown to have an influence on local immune functions as well [74-76].

Mice showed lower duodenal secretory IgA concentrations when they were subjected to prolonged intermittent fasting followed by an acute stressor [77]. Fecal IgA concentrations in anxious cats were higher when they were gentled (“petted”) meaning cats interacting positively with the caretaker at a shelter had higher fecal IgA concentrations than cats that responded negatively [78,79]. In adult dogs, levels of secretory IgA decreased following long-term stress [80] and acute stress [81,82]. In one study, male rats were kept in isolation and showed stable salivary IgA concentrations. When paired with a female, IgA concentrations showed an initial decline, followed by an increase over the next days indicating that introduction of a partner led to stress, but overall positively affected welfare [83]. Forced excessive and strenuous exercise in rats reduced secretory IgA and left individuals more susceptible to disease [83,84]. In racehorses, similar to human elite athletes, the rate of respiratory disease was higher while they had decreased IgA production [84,85].

One problem in interpreting the results of secretory IgA is the fact that the immune system does not react uniformly following hypothalamic-pituitary-adrenal (HPA) axis activation. While it is often up-regulated following acute stress, it may be suppressed by chronic stress [86]. Also, the magnitude of changes in secretory IgA concentrations may depend on other individual factors and may not always be consistent following a defined type of stimulus [76].

One study reported a higher probability of developing infectious diseases, especially of the mucous membranes, if concentrations of IgA in the secretions were at low levels in dogs [87]. In humans, patients with IgA deficiency present with various pathological intestinal conditions and have a high prevalence of inflammatory, malignant, and infectious gastrointestinal disorders [88,89]. IgA deficiency has also been reported in certain dog breeds and is associated with a high susceptibility to mucosal infections [90,91]. Amongst other immune regulatory proteins IgA has an influence on the gut microbiome in humans and may therefore have an impact on disease penetrance of inflammatory bowel disease [92]. Dogs with inflammatory bowel disease also displayed lower fecal IgA concentrations which might predispose them to develop chronic enteritis [12].

Gastrointestinal diseases such as enterocolitis develop in horses during hospitalization and antimicrobial treatment [14]. The decrease of intestinal IgA after anaesthesia shown in the current study could be an important factor in the pathogenesis of gastrointestinal diseases such as enterocolitis and is indicative of a stress-related aetiology.

Since IgA concentrations decreased in all horses but only a small percentage of the hospitalized horses develop enterocolitis, other factors must be present for the disease to occur. To evaluate whether a horse could have a deprived immune system and therefore could be prone to develop stress-associated diseases, additional biomarkers are of growing interest. In the past, studies have mainly focused on biomarkers reflecting the activity of the HPA axis (e.g., glucocorticoids). While glucocorticoids have an influence on local immunoglobulin levels [93,94], their serum concentrations do not reflect the functional state of stress-associated decrease in local mucosal immunity [32,33]. Furthermore, fecal IgA can be sampled in a non-invasive manner, so the sampling process should neither have an impact on the animals nor the measured biomarker [94].

One limitation of the present study is the fact that horses did not defecate at defined time points and that delayed intestinal passage may intervene with fecal IgA concentrations. To minimize the impact of the sampling technique, it was aimed to perform sampling at the same time of the day as a circadian rhythm has been reported for secretory IgA [95]. Secretory IgA is secreted into the digestive tract and accumulates in the feces prior to defecation [28]. As evaluation of IgA in feces depends on fecal consistency and gut transit time, gastrointestinal conditions may influence concentrations. Another important factor may be anaesthesia as it influences gastrointestinal motility as has been shown in humans and horses [96,97]. Additionally, reduced feed and water intake, which can be either due to anorexia or part of the pre or post surgical management, or endotoxaemia may also play an important role in altering gut motility [98].

Another weakness of this study was the fact that it included variable diseases and duration of surgeries. While efforts have been made to categorize the horses according to disease type, duration of general anaesthesia and surgical trauma, this may have caused a bias.

Overall, fecal IgA determination in horses may serve as an additional biomarker to detect patients that are likely to develop stress-induced diseases such as enterocolitis in the future and could therefore help implement countermeasures like reducing stress or efforts to improve IgA production.

5. Conclusion

The study emphasizes on the need to monitor hospitalized equine patients as factors related with hospital treatment are associated with a high risk to develop gastrointestinal diseases. In future, fecal IgA measurement may be a method to evaluate gut immunity in a minimally invasive manner.

Ethical Statement

Hereby, I Anna May consciously assure that for the manuscript "Effect of hospitalization on equine local intestinal immunoglobulin A (IgA) concentration measured in feces" the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.
2) The paper is not currently being considered for publication elsewhere.
3) The paper reflects the authors' own research and analysis in a truthful and complete manner.
4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
5) The results are appropriately placed in the context of prior and existing research.
6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.
7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.
8) For all horses included in this study ethical approval was granted by the Bavarian Government Animal Ethics Committee (No. 55.2-1-54-2531.2-25/07).

CRediT authorship contribution statement

A. May: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **H. Gerhards:** Writing – review & editing, Resources, Project administration, Conceptualization. **B. Wollanke:** Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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