Received: 30 August 2021 | Accepted: 27 May 2022

DOI: 10.1111/jvim.16470

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Adverse effects of polymyxin B administration to healthy horses

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Funding information
American College of Veterinary Internal Medicine, Grant/Award Number: 569545; Stiftung Pro Pferd, Switzerland, Grant/Award Number: 2018-02

Abstract

Background: Polymyxin B (PolyB) is used to treat endotoxemia in horses; neurologic and nephrogenic adverse effects occur in humans.

Objectives: To describe PolyB adverse effects in horses.

Animals: Five healthy horses (ataxia 0/5), 1 horse with cervical osteoarthritis (ataxia 1/5).

Methods: Prospective blinded randomized cross-over trial; 3-weeks wash out. Horses received PolyB (PolyB 6000 IU/kg IV, 7 doses q12h, n = 6) and PolyB/gentamicin (PolyB 6000 IU/kg IV, q12h 7 doses; gentamicin 10 mg/kg IV q24h 4 doses n = 4, or q12-24 h 5 doses because of an additional erroneous dose, n = 2). Daily neurological examinations were video recorded, and ataxia graded by 3 observers. Urine status, urinary GGT/creatinine ratio, plasma creatinine, and urea were assessed every other day, EMG daily. Mixed model analysis was used to evaluate factors associated with ataxia grade and [PolyB].

Results: Median ataxia score increased from 0/5 (range 0-2/5) to 2/5 (range 1-3/5) during administration and declined to 0.5/5 (range 0-2/5) after cessation. Gentamicin co-administration ($P < .01$, effect size: .8), number of PolyB doses ($P < .001$, effect size: .6), and time since last PolyB dose ($P < .001$, effect size: .5) had a significant effect on ataxia grades, while horse, day, [Genta], [PolyB], and $[PolyB]_{CSF}$ did not. Gentamicin co-administration and [Genta] C_{peak} had no effect on median [PolyB] C_{peak} (4.67 and 4.89 μg/ml for PolyB and PolyB/gentamicin, respectively). Urinary GGT/creatinine ratio was elevated in 3/6 horses receiving PolyB/gentamicin. The EMG remained unchanged.

Conclusions and Clinical Importance: PolyB caused transient ataxia, worsening with cumulative PolyB doses and gentamicin co-administration. Nephrotoxicity of PolyB was only evident when gentamicin was co-administered.

Abbreviations: [Genta], plasma gentamicin concentration; [PolyB], serum polymyxin B concentration; $[PolyB]_{CSF}$, polymyxin B concentration in CSF; CI, confidence interval; C_{peak}, peak concentration; CSF, cerebrospinal fluid; C_{trough}, trough concentration; MDR, multidrug-resistant; MUAP, motor unit action potential; PolyB, polymyxin B.

KEYWORDS
aminoglycosides, ataxia, endotoxemia, gentamicin, nephrotoxicity, neurotoxicity

1 | INTRODUCTION

Endotoxemia is a common occurrence in horses with colic, diarrhea, neonatal sepsis, and other bacterial infections.[1-3] Systemic inflammation follows the exposure to bacterial toxins, impairs cardiovascular, coagulation, and immune systems and results in peripheral hypoxia.[4] Disease conditions associated with endotoxemia are a leading cause of morbidity and mortality in horses.[4-6] Polymyxin B (PolyB) is a peptide antibiotic binding to bacterial toxins and therefore reducing activation of the proinflammatory cascade.[5,7,8] There is improvement in clinical variables and reduction of inflammatory mediators in horses treated with PolyB.[5,9-11] Polymyxins are listed as critically important antimicrobials by the world health organization (WHO) because of their antimicrobial action against multidrug-resistant (MDR) pathogens.[12,13] The decision to treat a horse with PolyB should therefore be made after careful consideration of other possible treatment options and the presence of life-threatening endotoxemia.

Polymyxins are used in human medicine with increasing frequency because of their antimicrobial action against a wide range of gram negative bacteria; however, their use is restricted because of their severe nephro- and neurotoxicity.[13,14] Renal dysfunction and failure are the most common and important adverse effects of polymyxin treatment in human patients.[14] Polymyxins induce acute tubular damage, evidenced by increases in serum creatinine and urea concentrations in rat models.[15] The underlying mechanisms include accumulation of polymyxin in the tubular cells of the kidneys and induction of cellular cell cycle arrest and cell apoptosis via death receptors and mitochondrial pathways.[16,17] Factors which increase the risk of nephrotoxicity in humans are the duration of therapy, the dose administered, concomitant administration of other nephrotoxic medications, and preexisting renal disease.[14]

Neurotoxicosis, although less common, causes paresthesia and neuromuscular weakness by a neuromuscular blockade.[18] Reduced sensitivity of the motor end plate to acetylcholine is considered the main mechanism, but recently nerve damage because of oxidative stress was identified in a mouse model.[18,19] Neuromuscular blockade occurs with other antimicrobials including aminoglycosides, tetracyclines, or lincosamides.[20] Amplification of neuromuscular blockade has been described in human medicine when co-administering aminoglycosides and lincosamides or aminoglycosides and calcium channel blockers.[21,22] Aminoglycoside antimicrobials, mainly gentamicin and amikacin, are routinely used in equine medicine, and their neurotoxic as well as nephrotoxic adverse effects could amplify the toxicity of PolyB when used concurrently.

Studies about adverse effects of PolyB in horses are sparse. No increase in urinary GGT/creatinine ratio was detected in studies evaluating the endotoxin-binding effect of PolyB.[9,10,23] Neurological adverse effects are not reported in these publications; however, preliminary data on weakness and ataxia after PolyB administration in endotoxemic horses exists.[24] At our hospital, similar clinical observations have been made (unpublished data).

The objective of this study was to describe the incidence, characteristics, severity, and duration of neurologic and nephrogenic adverse effects after PolyB administration to systemically healthy horses. The second objective was to assess possible influencing factors such as duration of PolyB administration, PolyB serum concentrations, and co-administration of gentamicin.

2 | MATERIAL AND METHODS

2.1 | Animals

Six warmblood geldings aged between 10 and 15 years were included in the study. Sample size was calculated to be a minimum of 4 horses per group to detect a difference in ataxia grade of 1/5 between 2 groups with an accepted alpha error of .05 and a beta error of .2 (https://clincalc.com/stats/samplesize.aspx, access 26 January 2018). Six horses were eventually included, as a mistake in gentamicin administration was made with horses 1 and 2.

All horses were judged to be systemically healthy based on clinical examination performed by a board-certified internal medicine specialist (AS), as well as normal blood work. A neurological examination was normal in all but 1 horse (horse 4), which showed an ataxia grade of 1/5 on real-time examination. Based on history and prior medical records this was judged to be because of cervical vertebral osteoarthritis and the horse was nevertheless included in the study.

2.2 | Study protocol

Experiments were performed at the University of Zurich. Including the pretrial period and monitoring after administration, each of the 2 study periods lasted 7 days with a wash out period of 3 weeks in between. During the study periods the horses were stabled at the study location, while the washout period was spent in the home barn. Experiments were performed in 3 pairs; all horses completed the full trial. Horses were randomly allocated to the order of drug administration in a stratified fashion (Figure 1). Horses received PolyB and then PolyB/gentamicin (horse 1, 2, 5, 6; $n = 4$), or PolyB/gentamicin, followed by PolyB (horse 3, 4; $n = 2$). The unequal distribution was because of a mistake in the study protocol for horses 1 and 2. Two additional horses (horse 5 and 6) were added to the study and assigned to the same drug administration sequence as horses 1 and 2. Horses 1 to 4 completed the study periods in alternating weeks: Horses 3 and 4 had their study period during the washout period of

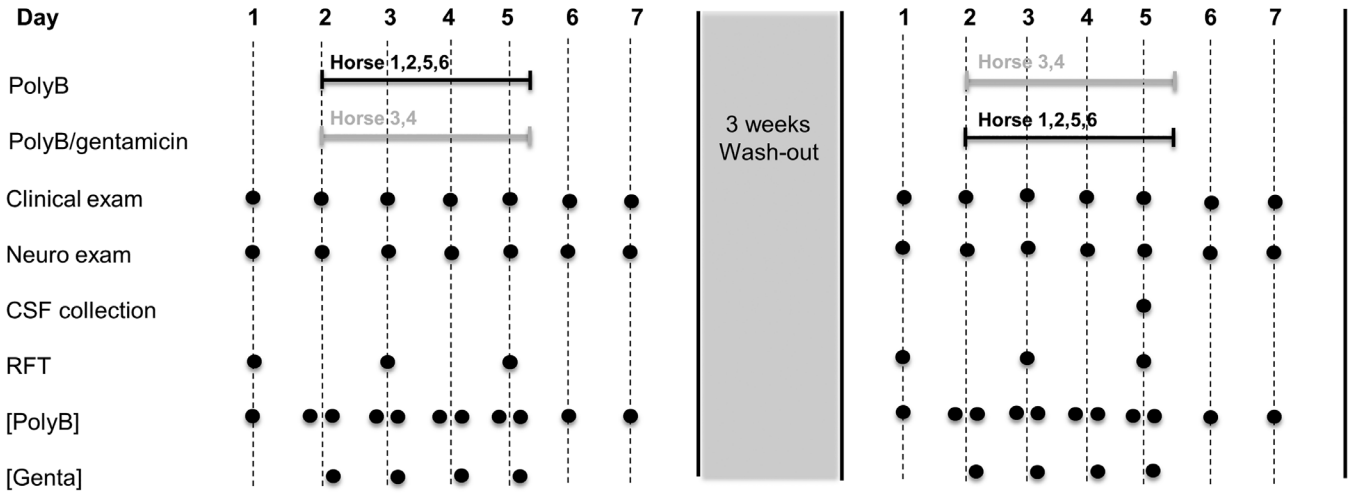


FIGURE 1 Study design of a cross-over prospective study evaluating adverse effects of polymyxin B in 6 systemically healthy horses with and without concurrent administration of gentamicin. PolyB: 7 doses polymyxin B 6000 IU/kg IV q12h. PolyB/gentamicin: 7 doses polymyxin B 6000 IU/kg IV q12h and 4 concurrent doses of gentamicin 10 mg/kg IV q24h. Clinical exam, full clinical examination; neuro exam, neurological examination recorded on video for later evaluation and EMG examination; RFT, renal function test comprised of urine analysis and plasma creatinine and urea measurement. [Genta] was only measured when the horses received PolyB/gentamicin

horses 1 and 2 and vice versa. Horses 5 and 6 entered the study 4 weeks after horses 1 to 4 had finished their trial, otherwise the study setup was the same. PolyB administration consisted of 7 doses of PolyB 6000 IU/kg IV q12hr (polymyxin-B-sulfate 100 000 IU/mL compounded by the Kantonsapotheke Zurich, ingredients for 1 mL: 100 000 IU Polymyxin-B-sulfate, NaCl, Conserv. 1 mg E218). PolyB/gentamicin administration consisted of 7 doses of PolyB 6000 IU/kg IV q12hr and 4 doses of gentamicin 10 mg/kg IV q24h (Genta 100, CP Pharma, Burgdorf Germany). Horses 1 and 2 erroneously received 1 additional gentamicin dose between the first and second dose (12 hours after the first dose on Day 2 of PolyB/gentamicin, 12 hours before the next dose on Day 3), resulting in a total of 5 doses of gentamicin. The gentamicin dose of 10 mg/kg q24h is routinely used at our hospital based on scientific data supporting this dose.[25,26]

Horses underwent a 2-hour transport from their home stable to the study location on the morning of Day 1. They were housed in individual boxes and fed hay at their home stable as well as at the study location. No exercise was performed during the study period. Horses were accustomed to the environment and the neurological examination, including electromyography measurement (EMG), for 24 hours before starting drug administration on Day 2. A venous catheter was placed into the jugular vein on Day 2. PolyB was administered at 10:00 am and 10:00 pm on Day 2, 3, and 4, and at 10:00 am only on Day 5. Gentamicin was administered at 10:00 am from Day 2 to Day 5 during PolyB/gentamicin administration. The catheter was removed on Day 5. A full clinical and neurological examination, including EMG, was performed daily while the horses received drugs and continued for an additional 2 days. The length of post-trial monitoring was based on the clinical experience that neurological signs resolve within 3 days after discontinuation of PolyB. Horses were afterwards returned to their home stable for the washout period or at the end of the trial.

The neurological examination followed a standardized protocol and videos of the examination were recorded for later evaluation using a standardized score sheet (Data S1 and S2, Supporting Information). Real-time neurological examination and ataxia grading using the ataxia scale 0-5 (Reed 2008) was performed by an internal medicine second year resident (JvS) to ensure that continued inclusion of the horses in the trial was possible. Examination of behavior, mentation, and cranial nerves was performed real-time by 1 examiner (JvS). This part of the neurological examination was not reviewed by the other observers as no abnormalities were noted in any of the horses at any time, and inter-rater agreement has been shown to be good.[26] Video sequences contained gait analysis walking in a straight line, with the head elevated, backing up, walking over obstacles, circling, tail pull, proprioception testing, standing sway test, spinal reflex testing (anal, cutaneous trunci), and cutaneous sensation testing. Each video was numbered and randomized before analysis to assure blinding of examiners. Evaluation of the video sequences was done by 3 examiners, including 1 ECVN neurology diplomate, 1 ECEIM equine internal medicine diplomate, and 1 ACVIM large animal internal medicine diplomate as this part of the neurological examination shows high inter-rater variability.[27,28] Ataxia was graded using the ataxia scale 0-5 and presence of ataxia was defined as a median ataxia score ≥1/5.[29] As this ataxia scale only provides crude neurological grading, an additional scoring system was used for better refinement. Observers were asked to grade deficits (none/slight/obvious) in each point of the analysis (gait analysis at walk, with elevated head, backing, over obstacles, circling, tail pull, proprioception testing, standing sway test, spinal reflex testing, and cutaneous sensation testing). Presence of weakness was recorded. Weakness was defined as ≥2 observers recording the presence of weakness in at least 1 test. Total deficits were scored by giving 1 point for slight deficits, 2 points for obvious deficits, and additionally 1 point for weakness, circumduction, and changes in spinal

reflexes and cutaneous sensation. A maximal score of 21 points was possible.

Electromyography (EMG) was performed once daily during the trial period by a board-certified neurologist (KB). The examiner was blinded to the drug administration protocol of the horses during EMG performance and evaluation. Depending on the temperament of the horses EMG was performed either with or without light sedation (xylazine .2-.4 mg/kg IV). EMG was performed using a NeMus 2 vet portable EMG device. Twenty-six-gauge concentric EMG needle electrodes were used. Band pass filter was set between 5 Hz and 10 kHz. Sweep speed was set between 20 ms/division. Amplifier gain was set between 50 and 100 μV for spontaneous activity and 500 μV for motor unit action potential (MUAP) recording. The following muscles were examined unilaterally: supraspinatus, deltoideus, biceps brachii, triceps brachii, and vastus lateralis. After insertion of the EMG needle spontaneous activity was recorded, then horses were manipulated to induce individual MUAPs and muscle fiber recruitment. The needle was moved to several locations within these muscles until approximately 20 different MUAPs could be identified. Abnormal spontaneous activity was considered present if it could be repeatedly identified in ≥2 locations per muscle. Analysis of the MUAPs was performed off-line. The MUAPs were selected and quantitively analyzed regarding amplitude, duration, and phases.

PolyB serum concentrations [PolyB] were measured 30 min before (C_{trough}) and 30 min after (C_{peak}) the first dose of the day (10:00 am, Days 2-5). Once PolyB administration was discontinued, [PolyB] was measured every 24 hours for an additional 2 days (10:00 am, Days 6 and 7; see Figure 1). Gentamicin plasma concentrations [Genta] were measured 30 minutes before the first dose (baseline) and 30 minutes after each administration (C_{peak}) which was given in the morning (10:00 am, Days 2-5 during PolyB/gentamicin administration). Blood was taken from the catheter or by venipuncture once the catheter was removed. Blood was collected in serum and heparinized blood tubes, and serum and plasma harvested. Both were stored at −4°C until all samples were collected (sampling time October 2018-February 2019) and measurements were performed in March 2019. PolyB concentration in the cerebrospinal fluid ($[PolyB]_{CSF}$) was measured once in every horse. Cerebrospinal fluid (CSF) was collected in the standing sedated (detomidine .01 mg/kg IV) horse via lumbosacral puncture 4 to 6 hours after the last PolyB dose irrespective of group allocation (Day 5 of the second study period for all horses, see Figure 1). Microprotein concentration and total nucleated cell count was measured within 1 hour after collection with the benzethonium chloride assay on a Cobas 6000 (Roche Diagnostics); total nucleated cell count was determined after staining with Samson solution in a Fuchs-Rosenthal counting chamber and a cytospin preparation was used for cell differential. For the measurement of PolyB concentrations, 3 mL of plain CSF were stored at −4°C until measurements were performed. PolyB measurements were performed in serum and CSF samples at the institute of forensic medicine of the University of Zurich using LC-MS (liquid chromatography-mass spectrometry). Gentamicin concentrations were measured in heparinized plasma by using fluorescence polarization on a COBAS INTEGRA 800 (Roche Diagnostics) by the Institute for Clinical Chemistry of the University of Zurich.

To assess and monitor kidney function, creatinine and urea were measured from heparinized plasma every second day (10:00 am on Days 1, 3, 5). On the same days, urinalysis including dip stick analysis, sediment analysis, specific gravity, creatinine concentration, and GGT activity from a free catch urine sample were performed by the Clinical Laboratory of the University of Zurich. Urine was collected free catch between 10:00 am and 8:00 pm. Creatinine concentration, urea concentration, and GGT activity were measured photometrically on a Cobas 6000 (Roche Diagnostics) using the kinetic colorimetric compensated Jaffé method for creatinine concentration (Roche Creatinine Jaffé Gen.2), a coupled enzyme reaction (Roche UREAL) for urea concentration, and an enzymatic colorimetric method (Roche GGT2) for GGT activity. Urine dip stick analysis was performed with Combur10 dipsticks read with a Cobas u411 (Roche Diagnostics), specific gravity was determined by refractometry, and sediment analysis was performed with 10 mL of fresh urine.

Withdrawal criteria were ataxia grade ≥4/5 in the real-time scoring or the presence of 2 or more of the following signs of renal function impairment: azotemia defined as creatinine >1.66 mg/dL (>147 μmol/L) or urea >53.5 mg/dL (>8.9 mmol/L), increase in serum creatinine >20%, urinary specific gravity <1020, proteinuria, urine casts, $creatinine_{serum}/creatinine_{urine}$ ratio < 37, or urine GGT/creatinine ratio > 25 IU/g.[30]

2.3 | Statistical analysis

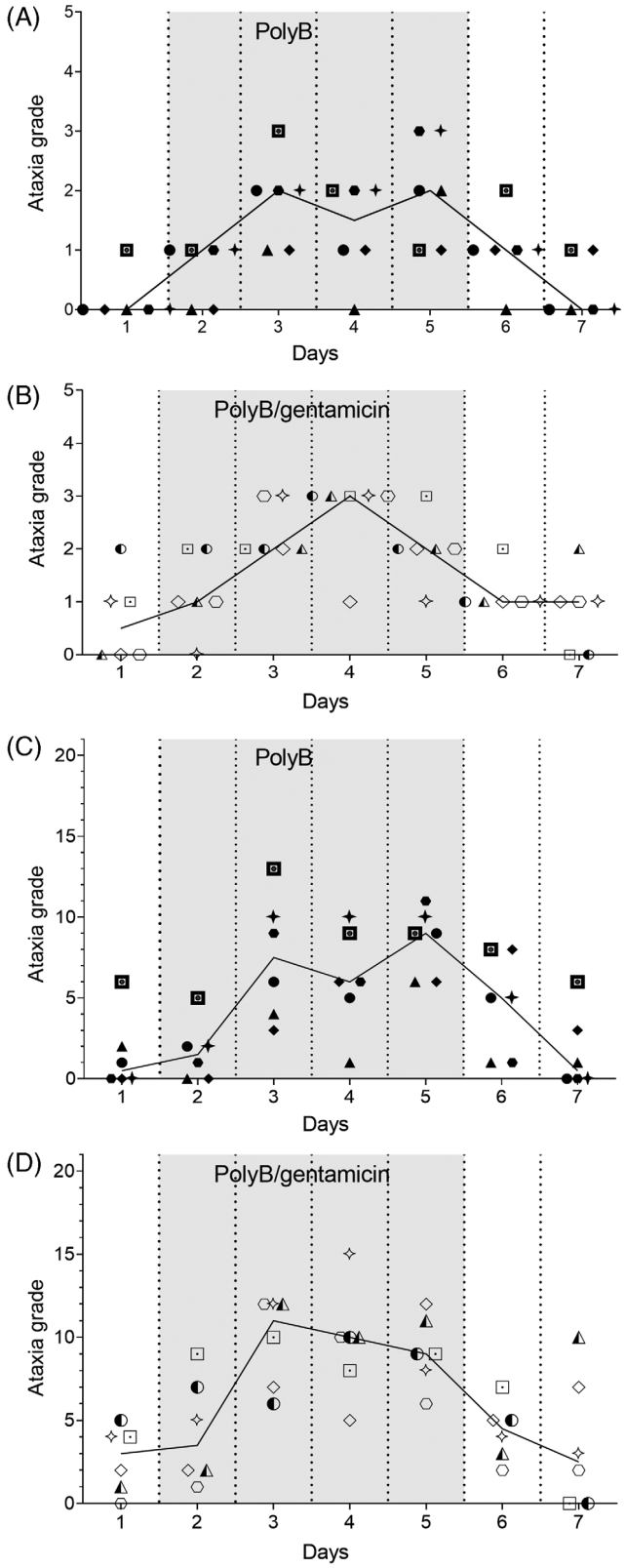
Normality of the data was tested by Shapiro-Wilk test. Because of non-normal distribution, data are presented as median and range, and non-normality was considered in the statistical analysis. A P value <.05 was considered statistically significant.

A Cumulative Link Mixed Model fitted with the Laplace approximation was used to evaluate the effect of several factors on the ataxia grade. Tested factors included individual horse number (1-6), day (Days 1-7), gentamicin co-administration (PolyB vs. PolyB/gentamicin), number of PolyB doses (0-7), time since last PolyB dose (0.5 hour for Days 2-5, 24 hours for Day 6, and 48 hours for Day 7), [PolyB] C_{peak}, [PolyB] C_{trough}, $[PolyB]_{CSF}$, and [Genta] C_{peak}. Gentamicin dosing (correct vs. erroneous) was added as variable to evaluate its effect and as it was not statistically significant, all 6 horses remained in the analysis. Effect size and proportional odds with 95% confidence intervals (CI) were calculated. A separate linear mixed model controlled for co-administration of gentamicin and time with individual horse number as random effect was used to evaluate the effect of gentamicin co-administration (PolyB vs. PolyB/gentamicin) and [Genta] C_{peak} on [PolyB] C_{peak} as dependent variable. Statistical analysis was undertaken in R (R Core Team 2020).

Inter-rater agreement was measured between observers by comparing all analyzed videos (n = 96). Krippendorff alpha was calculated to assess the level of agreement between observers for both scores

(0-5 and 0-21). Analysis was performed in R (R Core Team 2020). To assess intra-rater agreement, 12 videos were analyzed twice with different randomization number. Weighted kappa statistics were used to calculate intra-rater agreement of ataxia grading (score 0-5) on videos. An online calculator was used for analysis (https://www.graphpad.com/quickcalcs/kappa1/, access 24 January 2020).

EMG data were compared over time and between groups using the Kruskal-Wallis test. Statistical analyses were performed using a commercially available software package (IBM1 SPSS1 Statistics, version 25, 64-bit-version, IBM, Chicago, Illinois).



3 | RESULTS

All animals completed the study and the clinical examination remained normal throughout the study in all horses.

3.1 | Neurological effects

Real-time neurological examination showed normal mentation and normal behavior, without any cranial nerve deficits throughout the study in all horses. No horse had to be withdrawn from the study because of a real-time ataxia score ≥4/5.

All horses developed transient ataxia during PolyB and PolyB/gentamicin administration (Figure 2). In 65/84 (77%) of video scorings ataxia was present (grade range 1-3/5 and 1-15/21). The median ataxia score increased from 0/5 (range 0-2/5) and 1.5/21 (range 0-6/21) on Day 1 to 2/5 (range 1-3/5) and 9/21 (range 6-12/21) on Day 5. The median ataxia score of horses receiving PolyB increased from 0/5 (range 0-1/5) to 2/5 (range 1-3/5, peak on Day 5) and from 0.5/5 (range 0-2/5) to 3/5 (range 1-3/5, peak on Day 4) when receiving PolyB/gentamicin. The median neurological grade on the specially developed scoring system increased from 0.5/21 (range 0-6/21) to 9/21 (range 6-11/21, peak on Day 5) with PolyB and from 3/21 (range 0-5/21) to 11/21 (range 6-12/21, peak on Day 3) with PolyB/gentamicin. Ataxia grade declined to 0/5 (range 0-1/5) and 0.5/21 (range 0-6/21) after PolyB and to 1/5 (range 0-2/5) and 2.5/21 (range 0-10/21) after PolyB/gentamicin. On Day 7, all horses had decreasing ataxia grades compared to their peak ataxia grade.

FIGURE 2 Ataxia grading during a prospective cross-over trial evaluating adverse effects of polymyxin B in 6 systemically healthy horses with and without concurrent administration of gentamicin. Ataxia grading was performed using a scale 0-5 (A, B) and a scale 0-21 (C, D). Ataxia grading is the median score of three blinded observers based on video recordings. Each horse is indicated by a symbol. Lines represent the median. Shaded areas indicate days of drug administration. PolyB (A, C): 7 doses polymyxin B 6000 IU/kg IV q12h. PolyB/gentamicin (B, D): 7 doses polymyxin B 6000 IU/kg IV q12h and 4 concurrent doses of gentamicin 10 mg/kg IV q24h. Horse number 1 and 2 erroneously received an additional gentamicin dose (10 mg/kg IV) after 12 hours on Day 2 of the trial; the horses are marked by half filling of symbols. Horse 4 had a baseline ataxia scoring of 1/5 (based on real-time scoring) because of known vertebral osteoarthritis (marked by a dot in the symbol)

TABLE 1 Results of a Cumulative Link Mixed Model evaluating the effect of different factors on the ataxia grading (grade 0-5 and grade 0-21) during a prospective cross-over trial evaluating side effects of polymyxin B in 6 systemically healthy horses with and without concurrent administration of gentamicin

	Ataxia grading 0-5		Ataxia grading 0-21	
Factor	***P* value, effect size**	**Proportional odds (confidence interval)**	***P* value, effect size**	**Proportional odds (confidence interval)**
Gentamicin co-administration (PolyB vs. PolyB/gentamicin)	$P < .01, .80$	3.94 (1.62-9.46)	$P < .01, .74$	2.89 (1.32-6.58)
Number of PolyB doses	$P < .001, .64$	1.75 (1.38-2.21)	$P < .001, .64$	1.75 (1.41-2.17)
Time since last PolyB dose	$P < .001, .47$	−1.11 (−1.06 to −1.15)	$P < .001, .48$	−1.11 (−1.06 to −1.15)
[PolyB] C_{peak}	Not significant ($P > .05$)		Not significant ($P > .05$)	
[PolyB] C_{trough}	Not significant ($P > .05$)		Not significant ($P > .05$)	
$[PolyB]_{CSF}$	Not significant ($P > .05$)		Not significant ($P > .05$)	
[Genta] C_{peak}	Not significant ($P > .05$)		Not significant ($P > .05$)	
Individual horse	Not significant ($P > .05$)		Not significant ($P > .05$)	
Day	Not significant ($P > .05$)		Not significant ($P > .05$)	
Gentamicin dosing correct vs. erroneous	Not significant ($P > .05$)		Not significant ($P > .05$)	

In 24/84 (29%) video scorings weakness was detected with a peak of 6/12 (50%) weak horses on Day 5 of the trial and 2/12 (17%) on Day 1, 3/12 (25%) on Day 2, 4/12 (34%) on Day 3, 5/12 (42%) on Day 4, 2/12 (17%) on Day 6, and 2/12 (17%) on Day 7. All weak horses were also ataxic. Weakness was equally often detected with PolyB (12/42, 29%) and PolyB/gentamicin (12/42, 29%). None of the horses was judged to have a change in skin sensitivity and only in 1/84 exams 2/3 examiners agreed on the presence of reduced spinal reflexes. A video recording of a horse developing ataxia can be found in Data S2.

Factors with a statistically significant effect on the ataxia grade (score 0-5 and score 0-21) were co-administration of gentamicin (PolyB vs. PolyB/gentamicin), number of PolyB doses, and time since last PolyB dose (Table 1). The proportional odds of an increase in ataxia grading due to gentamicin co-administration (PolyB/gentamicin vs. PolyB) was 3.94 (CI 1.62-9.46) and 2.89 (CI 1.32-6.58) with the ataxia grading scales 0-5 and 0-21, respectively. With every dose of PolyB the proportional odds of an increase in ataxia grade were 1.75 (CI 1.38-2.21) for grading scale 0-5 and 1.75 (CI 1.41-2.17) for grading scale 0-21. With each unit increase in time since the last PolyB dose, the proportional odds of an increased ataxia grade were −1.11 (CI −1.06 to −1.15) for both grading scales. There was no effect of [PolyB] C_{peak}, [PolyB] C_{trough}, $[PolyB]_{CSF}$, [Genta] C_{peak}, horse number, or day of trial.

On EMG no abnormal spontaneous activity was detected in any muscle. No statistically significant difference was found within and between groups (PolyB vs. PolyB/gentamicin) or over time (all $P > .05$; Data S3).

3.2 | Effect on kidney function

None of the horses showed signs of nephrotoxicosis which would have precluded further inclusion in the trial. 3/6 (50%) horses receiving PolyB/gentamicin had elevated urinary GGT/creatinine ratio

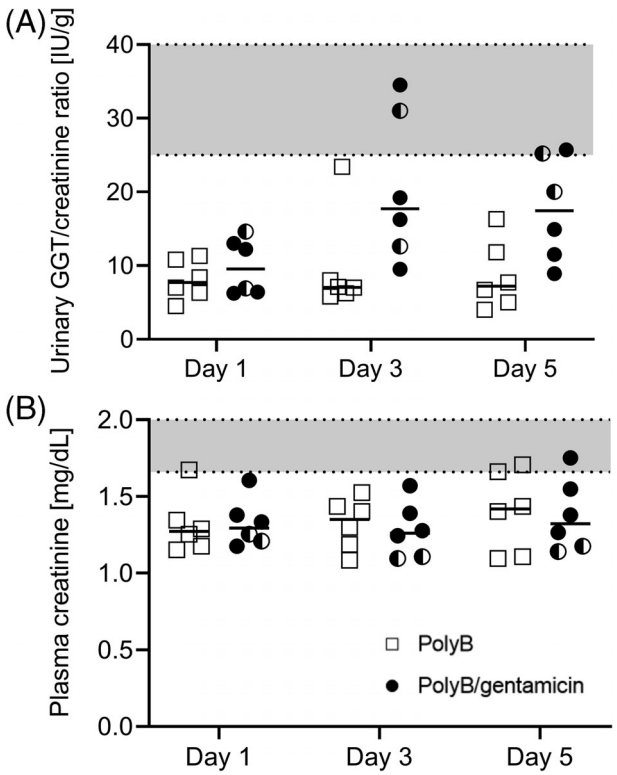


FIGURE 3 Urinary GGT/creatinine ratio (A) and plasma creatinine measurements (B) during a prospective cross-over trial evaluating adverse effects of polymyxin B in 6 systemically healthy horses with and without concurrent administration of gentamicin. PolyB (squares): 7 doses of polymyxin B 6000 IU/kg IV q12h. PolyB/gentamicin (circles): 7 doses polymyxin B 6000 IU/kg IV q12h and concurrent 4 doses gentamicin 10 mg/kg IV q24h. Shaded areas indicate values above the reference range. The horizontal line indicates the median. Half filling of symbols indicates two horses which erroneously received an additional gentamicin dose (10 mg/kg IV) on Day 2 of the trial

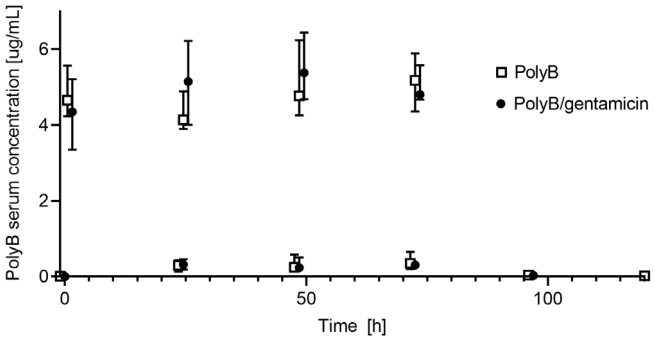


FIGURE 4 Serum concentrations of polymyxin B during a prospective cross-over trial evaluating adverse effects of polymyxin B in 6 systemically healthy horses with and without concurrent administration of gentamicin. PolyB (squares): 7 doses of polymyxin B 6000 IU/kg IV q12h. PolyB/gentamicin (circles): 7 doses polymyxin B 6000 IU/kg IV q12h and concurrent 4 doses gentamicin 10 mg/kg IV q24h. Two horses erroneously received an additional gentamicin dose (10 mg/kg IV) on Day 2 of the trial. Median and range are shown starting from time point 0 when the first polymyxin B dose was given

(Figure 3). One of the 2 horses which received an erroneous additional gentamicin dose on Day 2, showed an elevated GGT/creatinine ratio on Day 3 and Day 5, while the rest of the urinary analysis was within normal limits. A mild increase (<20%) in plasma creatinine concentration on Day 5 compared to Day 3 from 1.4 to 1.7 mg/dL (127-151 μmol/L) was seen in 1 horse receiving PolyB and in another horse from 1.6 to 1.8 mg/dL (from 139 to 155 μmol/L) receiving PolyB/gentamicin. There were no changes in the urine sediment and dip stick analysis in any horse. Urinary protein/creatinine ratio and $creatinine_{serum}/creatinine_{urine}$ ratio were within normal limits in all horses during both study periods.

3.3 | Polymyxin B concentrations

Median [PolyB] C_{peak} was 4.67 μg/mL (range 3.9-6.24 μg/mL) and 4.89 μg/mL (range 3.35-6.44 μg/mL) during PolyB and PolyB/gentamicin administration, respectively (Figure 4). Median [PolyB] C_{trough} concentration was .3 μg/mL (.13-.65 μg/mL) during PolyB administration, and .27 μg/mL (.19-.5 μg/mL) during PolyB/gentamicin administration. After discontinuation of drugs, [PolyB] was low in all horses (median .02 μg/mL, range .0-.06 μg/mL). There was no statistically significant effect of gentamicin co-administration or [Genta] C_{peak} on [PolyB] C_{peak}. Gentamicin concentrations can be found in Data S4.

3.4 | CSF analysis

CSF was obtained 4 to 6 hours after the last PolyB dose of the second study period. CSF could not be collected from 1 horse because of technical difficulties. Analysis was normal for all 5 samples (microprotein <.1 g/dL or < 1 g/L, leukocytes <5 cells/dL). Median $[PolyB]_{CSF}$ after the last dose of PolyB was .02 μg/mL and ranged from .0-.06 μg/mL.

3.5 | Rater agreement

Krippendorffs alpha was .53 (CI .45-.60) for the score 0-5 and .61 (CI .54-.68) for the score 0-21. Results therefore showed moderate agreement between raters with both scores. The score 0-21 showed a significantly better inter-rater agreement with a value above the upper CI for the score 0-5. Weighted kappa of intra-rater agreement of ataxia scoring (grade 0-5) by video analysis was .5, .7, and .9.

4 | DISCUSSION

This study showed that PolyB administration causes mild to moderate, transient ataxia in healthy horses. Duration of drug administration and co-administration of gentamicin exacerbated severity of ataxia. Acute renal dysfunction was not seen; however, several horses concurrently receiving gentamicin had an elevated urinary GGT/creatinine ratio as indicator of tubular cell damage.

Clinical signs of neurotoxicosis of PolyB in humans are paresthesia, neuromuscular blockade, muscle weakness, respiratory apnea as well as ataxia.[18] While paresthesia cannot easily be recognized in horses, clinical signs of muscular weakness and ataxia were seen in the horses in this study. Neuromuscular blockade causes lower motor neuron paresis and could therefore explain the weakness observed in 50% of the horses in our study.[31] However, proprioceptive ataxia as observed in all horses in this study indicated an additional sensory system abnormality. Transient ataxia after polymyxin administration is not only reported in humans, but development of transient ataxia is also described as an adverse effect in calves treated with high doses of PolyB intramuscularly.[18,32] Furthermore, preliminary data on horses show transient ataxia in 6/18 horses receiving PolyB treatment.[24] Unfortunately, the full data set is not yet published, precluding direct comparison to this study. Ataxia was also observed in rodents after polymyxin E application and severity of ataxia was correlated with electrophysiological and ultrastructural changes of the peripheral nerves.[33] In this rodent model, ataxia was recognized 3-5 days after initiation of drug administration and electrophysiological assessment identified a significant reduction in the amplitude of action potentials 7 days after.[33] Similarly, in our study, ataxia was recognized within the first days of drug administration, but EMG still failed to demonstrate significant changes in the first 6 days. EMG is considered a sensitive test for lower motor neuron diseases in horses.[34,35] Nevertheless, detection of abnormal MUAPs in neuromuscular blockade depends on the severity of the disease.[36] It is possible that the neuromuscular blockade was not severe enough to be detected by EMG. In the acute axonal injury that is expected in PolyB neurotoxicosis, MUAP morphology initially remains normal, only decreased recruitment patterns can be identified.[34] This could explain the normal EMG results.

The pathophysiology of neurotoxicosis of polymyxins is still incompletely understood. Earlier studies proposed presynaptic blockade of acetylcholine release, calcium induced prolongation of depolarization, and interaction with neuronal lipids as possible mechanism of action.[18,19] More recent experimental studies in rodents, however, indicate that mitochondrial dysfunction causes neurotoxicosis.[19]

Co-administration of muscle-relaxants, sedatives, or anesthetic drugs increases the risk of neurotoxicicosis in humans.[18] Little is known about drug interactions in horses; however, co-administration of neostigmine was associated with the risk of ataxia in horses receiving PolyB.[24] Neurotoxic effects of aminoglycosides are well known and include ototoxicity, peripheral neuropathy, encephalopathy, and neuromuscular blockade.[37,38] As gentamicin is a routinely used drug in equine medicine, we investigated its potential of amplifying neurotoxic adverse effects of PolyB and were able to show that ataxia was more severe during co-administration of gentamicin.[38] Presynaptic inhibition of acetylcholine release and post-junctional receptor binding by aminoglycosides possibly increase the neuromuscular blockade effect of PolyB. However, as newer studies indicate that polymyxin induced neurotoxicosis is rather caused by mitochondrial dysfunction than neuromuscular blockade, other mechanisms of aminoglycoside neurotoxicity such as oxidative stress, lysosomal abnormalities, and activation of NMDA (N-methyl D-aspartate) receptors need to be considered.[19,37] Two horses had a higher baseline ataxia score on video analysis when receiving PolyB/gentamicin compared to PolyB, while the real-time score was 0/5 before drug administration. Video-based scores showed a high variability between the 3 observers with a range of 0-2/5 in both horses. When reviewing the videos, it became evident that these horses were very nervous and agitated during the examination. Stress and habituation have shown to influence gait analysis in horses, and this might have influenced results.[39] Because of financial and organizational reasons, horses were accustomed to their new environment only for 1 day. As it was the second study period and therefore second stay at the study location for both horses, stress because of environmental changes is considered rather unlikely, but other unknown stressors might have been present. The difference in baseline ataxia scores could have introduced some bias on the overall effect of gentamicin on the development of ataxia.

Higher polymyxin dose as well as a longer duration of therapy increase the risk and severity of adverse effects in humans.[14] Likewise, we could demonstrate the effect of duration of PolyB administration on the ataxia grade in horses. PolyB serum concentration, however, was not correlated with neurological adverse effects. Binding of polymyxins to tissues high in lipids such as brain and muscle has been suggested to explain the neurotoxicity, however, newer studies have shown low [PolyB] in brain tissue using newer sensitive and specific methods.[40-42] To detect PolyB concentrations in the ventricular system, CSF was sampled in our study, but CSF concentrations were low and did not correlate with ataxia scores. Neuropathologic changes seen in mice after polymyxin therapy are reversible and signs in humans usually subside after prompt cessation of the drug.[18,33] Accordingly, our results showed a decreasing grade of ataxia with increasing days after the last dose.

Acute kidney injury occurs in up to 60% of human patients receiving PolyB but was not present in our study and previous studies on PolyB use in horses.[9,10,23,43] Doses up to 25 000 IU/kg/day are needed to reach an antibiotic effect in humans, compared to lower dose of 5000-15 000 IU/kg/day to reach anti-endotoxic effects in horses.[9,23,44] This could explain the discrepancy. The nephrotoxicity of PolyB should be further studied in a larger cohort, including horses with severe illness that compromises renal function. Besides neurotoxicity, aminoglycosides also share nephrotoxicity with polymyxins. An increase in the urinary GGT/creatinine ratio has been described in horses receiving systemic gentamicin.[30] Similarly, we saw an increase in urinary GGT/creatinine ratio in horses receiving PolyB/gentamicin. Both, polymyxins and aminoglycosides, accumulate in the proximal tubule cells and possibly lead to cell death via several different mechanisms and synergistical toxic effects seem likely.[14] It is unknown whether the seen increase in urinary GGT/creatinine ratio is caused solely by the relatively high dose of gentamicin alone or due to the combination of both drugs.

Several limitations need to be considered when interpreting the results of the present study. Evaluation and grading of an ataxic horse are difficult and individual experience and knowledge might influence results. We tried to overcome this by including 3 individual blinded observers with different experience. Although studies in horses showed a high agreement between observers deciding if a horse was ataxic or not, only in 20% of horses a good agreement for the grading of abnormalities was reached.[28] Inter-rater agreement in the present study was moderate and increased by using our newly developed ataxia score (0-21). The goal was to find a score which would allow smaller steps in neurological grades to detect smaller differences between groups or over time. When results indicated higher inter-rater agreement, this was rather surprising as one would expect lower numbers of agreement the more option raters have. The newly developed score needs to be validated in other populations of horses and raters but might be a useful ataxia grading system. An objective assessment of ataxia and lameness by using kinetic gait analysis has been developed in horses, but the needed equipment is not widely available.[45] Analysis based on video recordings might have impeded neurological grading but real-time grading by multiple examiners was not feasible.

The high level of inter-subject variance of EMG measurements and sensitivity of this method is also dependent on the experience of the investigator which might have influenced EMG results as only a single person performed the exam in this study.[46]

A small sample size was used with only 6 included horses. The power of the study was however sufficient to demonstrate development of transient ataxia and the influence of number of doses and gentamicin co-administration. Gentamicin dosing (correct vs. erroneous) was added as a variable into the statistical model and was not significant, therefore, we elected to leave the 2 erroneously dosed horses in the analysis. It could be argued that low power could

have resulted in this factor not showing up as significant in the analysis and different dosages could also have had an influence on other factors not reaching significance. However, this does not change the overall results. It was not the goal of the study to evaluate the influence of different gentamicin dosages but only the presence of gentamicin which was directly compared to the absence of it.

Care must be taken when extrapolating results of this study to clinical patients. Equine patients in need of PolyB treatment are typically endotoxemic and therefore severely ill horses with other organ systems affected. Patients with impaired renal function or myasthenia gravis have increased risks of showing signs of PolyB toxicosis, but subtle signs could also be overlooked in severely sick patients.[37] Further studies are needed to report and analyze PolyB toxicity in sick horses.

This study showed the presence of neurotoxicosis in healthy horses receiving 7 doses of PolyB 6000 IU/kg IV q12h. Ataxia was mild to moderate and reversible after cessation of therapy. Number of PolyB doses and co-administration of gentamicin increased the severity of ataxia. Gentamicin co-administration should be considered as an influencing factor for neuro- and nephrotoxicity. Clinicians need to consider all influencing factors to make a risk assessment and decide about the therapy of an individual horse to avoid toxicity whenever possible. PolyB is listed as critically important drug for use against MDR bacteria in human medicine and its use should be reflected weighting the need of an individual horse against one health considerations.

ACKNOWLEDGMENT

This paper has been supported by a research grant from the American College of Veterinary Internal Medicine (ACVIM) (number 569545) and a grant from Stiftung Pro Pferd, Switzerland (number 2018-02). Subject was presented as an abstract at the 2020 ACVIM Forum On Demand. The authors thank Prof Dr P. Torgerson and Henning Richter for their help with the statistical analysis and Dr Montavon for the cooperation to use horses of the Swiss national center in Berne.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Polymyxin B and gentamicin were used off-label in this study. Polymyxin B was used at a dose of 6000 IU/kg IV q12h with the indication of endotoxin binding. The dose used for gentamicin was 10 mg/kg IV q24h.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was performed under the regulations of the Swiss federal authorities for animal experimentation (animal use license no. ZH 022/18).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

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How to cite this article: van Spijk JN, Beckmann K, Wehrli Eser M, et al. Adverse effects of polymyxin B administration to healthy horses. *J Vet Intern Med*. 2022;36(4):1525-1534. doi:10.1111/jvim.16470