INTRODUCTION

Hepatic encephalopathy (HE) is a complex disease that is currently not fully understood. One of the presumed key factors in the pathogenesis of HE is ammonia. Ammonia is produced by bacterial deamination of amino acids in the gastrointestinal tract. Ammonia diffuses into the portal circulation and is delivered to the liver, where it is detoxified via the urea cycle. When liver function is impaired or when portal blood bypasses the liver, ammonia can accumulate in blood. Hyperammonemia is toxic to the central nervous system (CNS) because it alters amino acids (ie, glutamate and glutamine), neurotransmission systems (ie, gamma-butyric acid), nitric
oxime synthesis, and signaling transduction pathways, channels, and transporters. Furthermore, hyperammonemia could lead to central energy deficits (i.e., alpha-ketoglutarate), oxidative stress, and impairment of axonal and dendrite growth.\(^1\)\(^2\)

Although blood ammonia concentrations are frequently measured in people suspected of HE, these systemic levels do not necessarily reflect the levels in the CNS.\(^8\) The permeability of the blood-brain barrier (BBB) to ammonia is increased in human patients with HE secondary to liver disease, which could induce HE even if circulating ammonia concentrations are normal.\(^9\) Likewise, in dogs with portosystemic shunts (PSS), two research groups independently suggested that ammonia concentrations in cerebrospinal fluid (CSF) are higher than those of healthy individuals.\(^10\)\(^11\) In the presence of HE secondary to PSS, a strong positive correlation between ammonia concentrations in blood and CSF is present.\(^11\) Although the exact ammonia concentrations in CSF could be irrelevant in most clinical situations, this knowledge might be of interest when monitoring treatment responses in HE patients. However, current methods to determine ammonia in CSF are laborious, expensive, and require advanced equipment and expertise.\(^12\) Moreover, a major limitation of these methods is that they are not intended for testing individual samples.\(^13\) Therefore, the aim of the current technical report was to compare the accuracy and precision of ammonia measurements in CSF using two commercial laboratory devices validated only for blood.

## 2 | MATERIALS AND METHODS

### 2.1 | Cerebrospinal fluid collection and storage

Cerebrospinal fluid of horses without neurologic disorders, euthanized for reasons unrelated to this study, were used. Immediately after euthanasia, CSF was collected from the cerebellomedullary cistern in BD (Becton Dickinson) vacutainers without additive. Only macroscopically normal CSF, with a microscopically determined cell count within normal limits (i.e., <5 white blood cells/µL), was stored. Vacutainers were placed in a cool bag containing frozen gel packs immediately and then stored at −20°C for a maximum of 75 days. The minimum amount of CSF necessary to perform a series of 12 serial dilutions was calculated to anticipate potentially high measurement variation, and CSF was collected until this amount was reached.

### 2.2 | Preparation of spiked cerebrospinal fluid samples

The evening before comparative evaluations were performed, CSF was thawed slowly overnight at 4°C. The next morning, the CSF of all horses was pooled and kept in a closed container stored in a cool bag containing frozen gel packs.

Concentrated ammonia (Ammonia 28%-30%, Merck EMD Millipore) was used to spike at the selected ammonia concentrations in the CSF pool. Six serial dilution sets (1200, 600, 300, 150, 75, 37.50, 18.75 µmol/L) were prepared, and, for each set, a blank pooled CSF sample served as the baseline control. As a consequence, per each set of serial dilutions, eight concentrations were tested. One serial dilution was prepared at a time. During the preparation of the serial dilutions, all samples were stored on dry ice and kept in capped tubes to minimize exposure to air until their analyses were performed.

### 2.3 | Quantification of ammonia in cerebrospinal fluid

Two commercial blood ammonia analyzers (Catalyst Dx [CatDx], IDEXX laboratories and Pocket Chem BA [PocBA], A. Menarini Diagnostics) were compared to measure ammonia concentrations in the pooled equine CSF spiked with ammonia. Both devices use a microdiffusion-based colorimetric assay. More specifically, test strips are impregnated with an alkaline borate buffer that converts any dissolved ammonium (NH\(_4\)\(^+\)) in the sample into gaseous ammonia (NH\(_3\)). Ammonia then diffuses across a selectively permeable membrane and reacts with a pH indicator. After using the appropriate incubation times indicated by the manufacturer, the degree of color development is proportional to the ammonia concentration in the sample. The analyzers differ in the pH indicator used, that is, bromophenol blue for the CatDx and bromocresol green for the PocBA.

The CatDx is a nonportable chemistry analyzer calibrated at 21°C; therefore, analyses were performed in an air-conditioned room at a stable temperature. As ammonia is released during the clotting process, it is recommended to measure ammonia in plasma or whole blood rather than in serum according to the manufacturer's guidelines. To measure ammonia in CSF, the sample type "other" was selected on the analyzer. Before running a new sample, the ammonia slide (test slide) used for the previous sample was removed, and the analysis of a new series was only started when all samples from the previous series were removed from the machine. The latter measurements were taken to prevent ammonia from the slides or remaining sample from influencing the measurements of the next sample (minimize risk for carry-over). The detection range of ammonia in blood is 0-950 µmol/L. A sample volume of 300 µL is required per analysis.

The other device, PocBA, is a portable blood ammonia analyzer. According to the manufacturer's guidelines, whole blood is recommended for analysis. The detection range of ammonia in blood is 8-285 µmol/L. The reagent strip packages were opened just prior to use, and we carefully avoided touching the sample retention layer. A 20 µL sample volume was applied to the reagent strip.

The pooled and spiked CSF sample to be analyzed (320 µL) was placed in a sample cup for the CatDx, immediately after the dilution was prepared. The sample (20 µL) for the PocBA was taken from the sample cup using a capillary tube just before its insertion into the CatDx. Two operators (ND and HdR) performed the analyses in parallel with both devices. Per serial dilution set (6 in total), analyses were always started with the lowest concentration, and then, sequentially increasing concentrations were analyzed to minimize interference due to carry-over. The analysis time needed per sample was 3 minutes 55 seconds and 3 minutes 20 seconds for the CatDx and PocBA, respectively. All analyses were performed on the same day.
2.4 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc). Data were tested for normality using the D’Agostino & Pearson omnibus normality test. Correlations between the spiked concentrations and each of both laboratory analyzers were evaluated using the Spearman correlation tests. Wilcoxon matched-pairs signed-rank tests were used to compare values between the spiked concentrations and each of both laboratory analyzers. Bland-Altman plots were created to plot differences between each laboratory analyzer and the spiked ammonia concentration as well as between both laboratory analyzers. Bias (mean difference) and 95% (mean difference ± 1.96 SD) limits of agreement were calculated. The coefficient of variation (CV) was calculated to assess the precision of both laboratory devices. Each dilution of the six separate sets of serial dilutions was treated as an individual sample. \( P < .05 \) was considered statistically significant.

3 | RESULTS

CSF from six healthy horses was collected, frozen, and subsequently pooled upon thawing; a total volume of 760 mL of CSF was thus obtained within 65 days.

After spiking a sample series in this pool, the CatDx and the PocBA analyzers showed excellent correlations with the spiked ammonia concentrations \((r^2 = 0.89\) and 0.95, respectively\) (Figure 1). Nevertheless, the serial spiked ammonia concentrations differed significantly with the PocBA and CatDx values (both \( P < .0001 \) (Table 1). A more detailed evaluation with Bland-Altman plots (Figure 2A-C) showed that bias among the ammonia concentrations measured with the PocBA analyzer and the actual spiked ammonia concentrations were closer to zero than those measured with the CatDx analyzer, which implies that the PocBA measurements were more accurate than those of the CatDx. For CSF ammonia concentrations below 150 \( \mu \text{mol/L} \), mild overestimation was seen with the PocBA analyzer, whereas at higher ammonia concentrations (>150 \( \mu \text{mol/L} \)), mild underestimation was observed. In contrast, the CatDx analyzer underestimated the ammonia concentrations over the entire concentration range tested, and the difference became more pronounced with increasing ammonia concentrations.

The coefficient of variations (CVs) for the CatDx and PocBA analyzers ranged from 0.09 to 1.55 and from 0.03 to 0.35, respectively (Table 2). Measurements performed with the PocBA analyzer were, therefore, more precise than those performed with the CatDx analyzer. Moreover, ammonia concentrations <75 \( \mu \text{mol/L} \) in equine CSF could not be measured precisely with the CatDx analyzer (Table 2).

4 | DISCUSSION

The present technical report demonstrates that ammonia can be measured in equine CSF with the CatDx and PocBA commercial blood ammonia analyzers. Both devices generated ammonia concentrations
that correlated with the spiked ammonia concentrations. A similar degree of correlation for the CatDx and PocBA analyzers was expected since they have a microdiffusion-based colorimetric assay in common. Despite this correlation, the values measured with both devices differed significantly from the spiked concentrations. However, the mean difference between the spiked ammonia concentration and the concentration measured with the PocBA analyzer was smaller than that measured with the CatDx analyzer. Quantifications performed with the PocBA analyzer were more accurate overall. Moreover, in case of clinically relevant ammonia excess (i.e., >150 µmol/L), PocBA underestimated concentrations less than the CatDx analyzer, making the PocBA analyzer more reliable for monitoring patients with HE. The PocBA measurements were also more precise than those of the CatDx, especially for the low ammonia concentrations. To evaluate the precision of both laboratory devices better, more samples for each concentration should ideally be tested, and analysis should be performed over multiple days. Even though within-day precision, as evaluated in this study, gives a notion about repeatability of the results, assessment of both within-day and between-day precision would have added more value to the evaluation of precision for both devices.14

A significant advantage of the PocBA analyzer is that it is a hand-held device. Blood ammonia measurements are known to be challenging. Ammonium ions are labile and preanalytical errors can occur by ammonia formation from endogenous metabolites in stored samples or by environmental contamination.6,8 Directly measuring ammonia concentrations from CSF patient samples decreases the potential risk of preanalytical errors. The CatDx analyzer, on the other hand, can simultaneously measure additional clinical biochemistry variables on the same sample. However, this advantage does not outweigh the disadvantages of being a nonportable device that needs to be calibrated at 21°C and consequently requires a temperature-controlled laboratory. The sample volume required for the CatDx analyzer is 300 µL, which is 15-fold higher than for the PocBA analyzer. This, however, does not seem to be a clinically relevant difference. A total of 1 mL CSF/5 kg of body weight can be collected in animals; thus 300 µL is feasible, even in small animals. In patients where only a very small CSF sample can be collected, the PocBA analyzer has an advantage over the CatDx analyzer. Finally, analysis times of less than 4 minutes were similar for both devices, and thus, were not considered to be a discriminating factor.

One limitation of the PocBA analyzer was that it has a relatively low upper quantitation limit (285 µmol/L) compared with that of the CatDx analyzer (950 µmol/L). However, this approximatively three-fold narrower dynamic range could be readily overcome by appropriate sample dilution. Nevertheless, additional dilution adds to the costs and time of the analysis and increases the risk for measurement errors.15 Ammonia concentrations detected in the CSF of dogs with PSS and concurrent HE were reported to all be below 300 µmol/L.11 As a consequence, it appears that the detection limit of the PocBA analyzer would be sufficient to measure ammonia concentrations in most (if not all) clinical patients. In this technical report, preanalytical errors were minimized by collecting ammonia immediately following euthanasia and by direct cooling of the samples. Regarding the aspect of storage stability on ammonia concentrations in CSF patient samples, a previous study showed no significant change in ammonia concentrations when human CSF was stored at −20°C for up to 1 month.13 Ammonia concentrations measured in the blank samples of the CSF pool that served as baseline control were all very low. The one blank sample with an ammonia concentration of 67 µmol/L when measured the PocBA analyzer, revealed an ammonia concentration of 0 µmol/L when measured in parallel with the CatDx analyzer. The low ammonia CSF concentrations after storage of up to 75 days at −20°C suggests that the storage time did not affect the ammonia concentration. In plasma, it is known that ammonia formation is a continuous process; changes in ammonia concentrations are more impacted by storage duration than storage temperatures.16,17

For current validation purposes, equine rather than canine CSF was chosen, for the practical reason of enabling the collection of a relatively large volume of CSF within an acceptable time frame. However, it should be mentioned that for ammonia measurements, canine research dominates the veterinary literature. Cerebrospinal fluid characteristics in healthy dogs and horses, such as color and clarity, and the presence of white blood cells, are similar. The upper limit of the total protein content, however, is fourfold higher in horses compared with dogs (<100 mg/dL vs <25 mg/dL, respectively).18 Even though this is a significant difference, ammonia does not bind to proteins. Proteins also do not influence the conversion of dissolved ammonium ions into gaseous ammonia. Therefore, interspecies differences are not likely to influence ammonia concentrations, and we postulate that it is very likely that our data in equine CSF could be extrapolated for canine CSF. A recent clinical study from our group looking at dogs with extrahepatic PSS with CSF ammonia measurements and using the same devices as performed in the current study supported our hypothesis. In that study, a correlation between CatDx and PocBA analyzer measurements was also found, and the mean ammonia concentration in canine CSF measured with the PocBA analyzer (106.6 ± 55.6 µmol/L)

<table>
<thead>
<tr>
<th>Spiked ammonia concentration (µmol/L)</th>
<th>CatDx Mean (range) (µmol/L)</th>
<th>PocBA Mean (range) (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>5.50 (0-17)</td>
<td>39.33 (30-67)</td>
</tr>
<tr>
<td>18.75</td>
<td>11.33 (0-35)</td>
<td>58.00 (44-84)</td>
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<tr>
<td>37.50</td>
<td>15.67 (7-49)</td>
<td>74.17 (59-93)</td>
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<tr>
<td>75.00</td>
<td>25.00 (14-33)</td>
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<tr>
<td>150.00</td>
<td>67.83 (47-121)</td>
<td>171.33 (166-179)</td>
</tr>
<tr>
<td>300.00</td>
<td>116.17 (107-136)</td>
<td>265.40 (233-high)</td>
</tr>
<tr>
<td>600.00</td>
<td>286.00 (222-322)</td>
<td>NA</td>
</tr>
<tr>
<td>1200.00</td>
<td>602.33 (400-706)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Note: NA: not applicable because the upper limit of detection of PocBA is 285 µmol/L.

Abbreviations: CatDX, Catalyst Dx; PocBA, Pocket Chem BA.
FIGURE 2  Bland-Altman plots of the differences between the spiked ammonia concentrations and the concentrations measured with (A) the Catalyst Dx (CatDx); (B) the Pocket Chem BA (PocBA); and (C) both the PocBA and CatDx analyzers. For each plot the upper and lower dashed lines represent the 95% limits of agreement. The solid line indicates the mean difference (bias)
was consistently higher than that measured using the CatDX analyzer (60.1 ± 42.9 μmol/L). The fact that CSF ammonia can be measured with commercially available laboratory devices boosts further research. Studies used to gain insight into how ammonia should be measured to study the pathogenesis of HE have become more feasible.

This study documents that CSF ammonia quantified with commercial blood ammonia analyzers correlated with CSFs containing spiked ammonia concentrations. Values obtained with the PocBA analyzer were more accurate and precise than with the CatDX analyzer. Both the CatDX and PocBA analyzers are fast, easy-to-use, and inexpensive devices with PocBA being a hand-held analyzer. Future studies are needed to establish reference intervals for ammonia in the CSF of different species using these analyzers.

**DISCLOSURE**

The authors have indicated that they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this article.

**REFERENCES**


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