Turbidimetric-Kinetic Assay of Endotoxin in Rumen Fluid or Serum of Cattle Fed Rations Containing Various Levels of Rolled Barley

Yoshiko MOTOI, Tsutai OOHASHI(1), Hisashi HIROSE(5), Miyako HIRAMATSU, Sigeru MIYAZAKI, Shigeyoshi NAGASAWA(3), and Junkichi TAKASHI(2)

Feed Safety Research Division, (1)Systematic Diagnosis Research Division, National Institute of Animal Health, 3-1-1 Kannondai Tsukuba, Ibaraki 305, and (2)Department of Specialty Chemicals, Wako Pure Chemical Industries, Ltd., 4-5-13 Nihombashi, Chuou-ku, Tokyo 103, Japan

(Received 2 July 1992/Accepted 7 October 1992)

ABSTRACT. A new, automated turbidimetric-kinetic (ATK) assay was used to quantitate bacterial endotoxin in rumen fluid or in serum of Holstein steers. The ATK method used Limulus amebocyte lysate (LAL) reagent with added beta-glucan (LAL-ES) which improved specific sensitivity to endotoxin. Design of the feeding trial permitted comparison of endotoxin levels found during consumption of a basal ration with those higher levels detected at various times following the introduction of increasing percentages of rolled barley to that basal ration. Both serum and ruminal endotoxin levels were significantly higher in steers on the higher concentrate rations. Peak endotoxin levels were detected 20 days following the change to the highest concentrate ration which contained 60% barley. Endotoxin levels from both sources subsequently decreased. Ruminal endotoxin stabilized at about 10 times the level, and serum endotoxin stabilized at 2 to 4 times the level, of that previously found during feeding of the basal ration. Test protocol included sample dilution and heating in order to avoid the effects of endotoxin inhibitors. Recovery rates for added endotoxin to either serum or rumen fluid supernates ranged from 120% to 136%. Coefficient of variation for endotoxin concentration in serum was lower than 10%, and in rumen fluid only slightly higher. There was significant correlation between ruminal concentration as measured by the ATK method and an alternative chromogenic substrate assay procedure. Changes in endotoxin level in experimental steers were those predictable from experience with naturally occurring incidents of grain engorgement. The ATK assay appears to be an accurate and rather simple technique which will prove useful for experimental and clinical studies in the future. —KEY WORDS: cattle, concentrate-overfeeding, endotoxin, Limulus lysate, turbidimetric-kinetic assay.


Prior reports suggest that endotoxins derived from rumen bacteria play an important role in the pathogenesis of several diseases of cattle. These are the diseases such as bloat, lactic acidosis or sudden death syndrome which, in turn, have been associated with grain engorgement of the rumen [1, 5, 11, 12].

Nagaraja and coworkers [13] detected endotoxin in cell-free rumen fluid by titrating their lethal effect in mice. Greater toxicity was detected in rumen fluid of cattle fed grain, as opposed to those fed hay [14].

The Limulus amebocyte lysate (LAL) test has been used to detect endotoxin by the gelation reaction [8]. Using the LAL test, Dougherty [1] detected endotoxin in the blood of sheep or steers following experimentally grain engorgement. The endotoxin concentration was estimated by recording the highest sample dilution that still resulted in gelation.

Neither approach determined specifically, the quantitative level of bacterial endotoxin in the blood or in the rumen fluid of cattle. Recently, an automated turbidimetric-kinetic (ATK) assay system for endotoxin in blood has become commercially available [7]. This simple quantitative and specific system has been used successfully to assay endotoxin in human plasma [23], however, it has not yet been applied to measurement of endotoxin in the blood or rumen fluid of cattle.

The present study was an attempt to assay endotoxin in either bovine serum or rumen fluid, using the new ATK assay system with endotoxin-specific LAL. Changes in the endotoxin concentration were monitored in cattle fed either a basal fattening ration, or that basal ration augmented by the addition of rolled barley at varying levels.

MATERIALS AND METHODS

Animals: Four clinically healthy Holstein steers, fitted with ruminal fistulas, were used repeatedly throughout this study. Their mean body weight was 330±22 kg at the start of the trial, and 380±23 kg at
its conclusion.

**Nutritional design:** Steers were fed twice daily at 9 AM and at 3 PM, with 50% of their total daily allotment at each feeding. The composition, daily intake level and duration of feeding of four experimental rations were as described in Table 1. Experimental rations 2, 3 and 4 were fed sequentially, following the initial adaptation period of 60 days on basal ration 1.

**Sample collections:** Blood samples were drawn from the jugular vein into a pyrogen free tube. Rumen contents were aspirated through the fistula by suction applied to a stomach tube and were filtered through a double layer of pyrogen-free gauze, and finally centrifuged at 10,000 rpm for 30 min. Both serum and supernatant ruminal fluid preparation were stored at −20°C until tested.

Rumen contents were routinely obtained immediately prior to the 9 AM feeding. They were collected after 10, 17 and 24 days on Ration 2; after 5 days on Ration 3; and again after 4, 10, 24, 31, 37 and 60 days of feeding Ration 4.

Sampling of both blood and rumen contents was performed repeatedly during the final day of feeding for both Ration 1 and Ration 4, at time intervals as graphed in Figures 3 and 4.

**Reagents:** Lipopolysaccharide (LPS; Escherichia coli UKT-B, Wako Pure Chemical Ind., Ltd., Tokyo) was used as a standard endotoxin for assay, and was dissolved in pyrogen-free distilled water (Otsuka Pharmaceutical Co., Tokushima), as was unmodified LAL (Limulus Hs-test Wako, Wako Pure Chemical Ind., Ltd., Osaka). Endotoxin-specific LAL was prepared by dissolving Limulus test reagent in tris buffer (pH 7.4) containing a high concentration of beta-glucan. Chromogenic substrate (Endospecy Test, Seikagaku Kogyo Co., Ltd., Tokyo) [17] for the colorimetric Limulus test was used in that alternative assay procedure. Detoxi-Gel columns (Pierce Chemical Co. Rockford, IL., U.S.A.) was used to detect the substances other than endotoxin in rumen fluid, also capable of causing gel formation.

**Instrumentation:** An automated turbidimetric-kinetic analytical instrument (Toxinometer ET-201, Wako Pure Chemical Ind., Tokyo) [16] was used to measure endotoxin concentration [23].

**Analytical methods:** All glassware and other supplies were detoxified by heating at 250°C or by gamma irradiation. To remove endotoxin inhibitors, serum was diluted 1:10, and rumen fluid 1:100,000, respectively, with endotoxin-free distilled water. Samples were heated at 100°C for 10 min, then chilled immediately in an ice bath until assayed.

Each sample, prepared in this manner, was then mixed with an equal volume (100 μl) of LAL-ES solution and placed in the optical unit of the analysis module which consisted of a pre-warmed (37°C) glass tube, 10 mm in diameter. Once inserted, turbidity measurement was initiated immediately, and the time required for gel formation was calculated.

The standard endotoxin was dissolved in endotoxin-free distilled water to form a standard series ranging in concentration from 1 through 10^5 pg/ml, in 10-fold increments. An identical 100 μl volume of each concentration was then assayed in the ATK system to establish the standard curve.

**Recovery test:** The standard endotoxin was dissolved in serum or in rumen fluid after endotoxin inhibitors had been removed. Final concentration

![Fig. 1. Endotoxin standard curve of turbidimetric-kinetic assay. The standard endotoxin (Escherichia coli UKT-B) was dissolved in distilled water in a five different final concentrations.](image-url)
Table 2. Recovery of endotoxin added to bovine serum and rumen fluid

<table>
<thead>
<tr>
<th>Endotoxin concentration (pg/ml)</th>
<th>Recovery (%)</th>
<th>Endotoxin concentration (pg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>130.9</td>
<td>10</td>
<td>126.9</td>
</tr>
<tr>
<td>100</td>
<td>128.2</td>
<td>100</td>
<td>125.2</td>
</tr>
<tr>
<td>1000</td>
<td>136.5</td>
<td>1000</td>
<td>120.4</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>131.9±3.5</td>
<td>Mean±SD</td>
<td>124.2±2.8</td>
</tr>
</tbody>
</table>

Table 3. Reproducibility of the endotoxin assay in serum and rumen fluid

<table>
<thead>
<tr>
<th>Endotoxin concentration</th>
<th>Serum (pg/ml)</th>
<th>Rumen fluid (×10^2 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.39</td>
<td>5.69</td>
</tr>
<tr>
<td>2</td>
<td>1.18</td>
<td>5.44</td>
</tr>
<tr>
<td>3</td>
<td>1.10</td>
<td>6.53</td>
</tr>
<tr>
<td>4</td>
<td>1.27</td>
<td>5.69</td>
</tr>
<tr>
<td>5</td>
<td>1.18</td>
<td>5.44</td>
</tr>
<tr>
<td>6</td>
<td>1.42</td>
<td>6.84</td>
</tr>
<tr>
<td>7</td>
<td>1.32</td>
<td>5.44</td>
</tr>
<tr>
<td>8</td>
<td>1.26</td>
<td>7.18</td>
</tr>
<tr>
<td>Mean</td>
<td>1.27</td>
<td>6.03</td>
</tr>
<tr>
<td>SD</td>
<td>0.11</td>
<td>0.66</td>
</tr>
<tr>
<td>CV</td>
<td>8.66%</td>
<td>10.95%</td>
</tr>
</tbody>
</table>

were 10, 100 and 1,000 pg/ml. The gelation times were measured using LAL-ES with the toxinometer. Gelation times of standard endotoxin preparations were compared to those of treated samples when results were plotted logarithmically.

Reproducibility test: Reproducibility of the ATK assay was examined, using both serum and rumen fluid from cattle. The interassay variance was measured on 12 succeeding days, using standard endotoxin in a concentration of 2.5 pg/ml.

Removal test: Rumen fluid, pre-treated by the dilution/heating steps described, were passed through a Detoxi-Gel column prior to assay, using the LAL procedure. Gelation time for fluids, both before and after column treatment, was then compared. The endotoxin reduction rate was calculated from the following formula:

\[
\text{Percent Reduction} = \frac{(\text{Conc B}) \text{ minus } (\text{Conc A})}{(\text{Conc B})} \times 100
\]

Where: (Conc B) = concentration before column extraction.
(Conc A) = concentration after column extraction.

RESULTS

Using the ATK assay and specific LAL-ES, the standard curve derived for endotoxin in distilled water was confirmed to be a straight line (Fig. 1) when log gelation time in minutes (Gt) was plotted versus log endotoxin concentrations, ranging from 1 through 10^5 pg/ml.

In serum, the means of recovery rate various added concentrations of LPS were 130.9, 128.2 and 136.5%, respectively. In rumen fluid, the rates were 126.9, 125.2 and 120.4%, respectively (Table 2).

The coefficient variation of the endotoxin concentration in serum was 8.66%, but in rumen fluid it was 10.95% (Table 3).

The mean gelation time, standard deviation and coefficient variation for standard endotoxin in a concentration of 2.5 pg/ml were 51.5 min, 3.87 min and 7.54%, respectively. The interassay variance for gelation time of 2.5 pg/ml of standard endotoxin was stable over a period of 12 days.

The concentration of gelation activity in rumen fluid before endotoxin removal was 4,328 ng/ml, while after endotoxin removal it was 0.0052 ng/ml.
Consequently, endotoxin reduction rate for rumen fluid, when calculated by the previous formula, approached 100% (99.999%).

There was significant correlation between the endotoxin concentrations measured by ATK assay test and chromogenic substrate method \( r=0.91, y=1130+0.25x \) as graphed in Fig. 2.

Rumen endotoxin concentrations of cattle on Ration 4 were significantly higher \( (P<0.01) \) and had greater individual variation than did endotoxin concentrations of cattle on Ration 1, at all samplings. Prior to 9 AM feeding, the mean endotoxin concentration in rumen of cattle on Ration 4 was 4,497 ng/ml (with range 907 to 13,200) in contrast to a level of 427 ng/ml (with range 231 to 762) for cattle feeding Ration 1. The mean endotoxin level in the former cattle varied from about 2,000 to 7,000 ng/ml during the succeeding 24 hours, in contrast to an almost constant level in the latter steers (Fig. 3).

In steers fed Ration 1, the serum endotoxin level ranged from 1 to 2 pg/ml (mean = 1.7) and little individual difference was detected, as was the case with ruminal endotoxin. Conversely, serum endotoxin concentrations in steers on Ration 4 were higher, ranged from 3 to 4.5 pg/ml (mean = 3.9), and had greater individual variation. However, there were no definite changes in ruminal and serum endotoxin levels associated with intake of either ration (Fig. 4).

Changes in endotoxin levels were also monitored as steers were fed Rations 2, 3 and 4, sequentially, in
comparison to levels measured while consuming the basal ration 1. Mean ruminal endotoxin level gradually increased after feeding Ration 2, and increased further after Ration 2 was replaced with Ration 3. Ruminal endotoxin level increased markedly (54±12.5 μg/ml) 4 days after change to Ration 4, reaching a maximum value of 96 ± 35 μg/ml on day 20. Later, the ruminal endotoxin level fell until it reached a concentration about ten times that of steers fed Ration 1 on day 60, and remained stable thereafter.

Mean serum endotoxin level varied in a similar manner, reaching a maximum of 10.2±3.6 pg/ml after Ration 4 was fed. This coincided with maximum ruminal endotoxin value. Subsequently serum endotoxin gradually decreased, and stabilized when it reached about 2 to 4 times the level of Ration 1-fed cattle (Fig. 5).

**DISCUSSION**

In recent years, outbreaks of a disease attributable to overfeeding of grain to ruminant livestock, especially to feedlot cattle, have been described [1, 5, 11, 12, 21]. Nagaraja et al. [12, 15] confirmed the presence of endotoxin in phenol-water extract of rumen bacteria derived from cattle fed either hay and grain, as well as the presence of free endotoxin in cell-free supernate of rumen fluid. In mouse toxicity testing, the level of free endotoxin in rumen fluid of cattle fed grain could be at least twice as high as in those fed roughage. These reports assured that the feeding of concentrates, especially grain, enhances ruminal production of free endotoxin. However, there is thus far no information correlating this increase to increased level of endotoxin in serum. A technically accurate and objective method to detect endotoxin in biospecimens has been lacking. Currently, human medicine mainly employs the chromogenic substrate method [17] for quantitative analysis of endotoxin in blood. However, several drawbacks, such as technical difficulty and inappropriate processing of endotoxin inhibitor in blood, have been pointed out [19].

Recently, Yokota et al. [23] have established plasma endotoxin assay method using a turbidimetric-kinetic assay system which involves a simple and direct quantitative assay of endotoxin. The assay is based on gelation pathways of LAL, which are triggered by two distinct stimulants, namely endotoxin and β-glucan. The former activates Factor C and the latter, Factor G [9]. Therefore, gelation may be accelerated additively when both activators are present in the samples. However, in the presence of excess beta-glucan, the additive effect was abolished [20]. In other words, the LAL-ES which contains 1 mg/ml of beta-glucan is sensitive only to endotoxin. LAL, in comparison is activated by both endotoxin and beta-glucan [20]. In the present study, we attempted to develop a direct assay method for endotoxin in the serum and rumen fluid of cattle, using the ATK assay system and LAL-ES, as described.

Pretreatment by dilution/heating not only removes endotoxin inhibitors but also recovers endotoxin activity [23]. Therefore, we diluted serum samples 10-fold and heated them at 100°C for 10 min. No reports either on inhibitors of rumen fluid or on methods to remove them are available. Empirically, the high dilution of 1:100,000 was
chosen as a suitable means to measure endotoxin in the absence of inhibitors in rumen fluids.

It was confirmed that almost all substances in rumen fluid capable of causing gel formation were endotoxin by the removal test using Detoxi-gel columns.

The standard curve for the ATK assay method is linear over a wide range of endotoxin concentrations from 1 to $10^5$ pg/ml and is sufficient for practical determinations of both serum and rumen fluid endotoxin levels.

The recoveries of endotoxin from serum and rumen fluid were more than 100 percent. A similar phenomenon in recovery from plasma has been reported. However, the exact mechanism in this phenomenon has yet to be elucidated, although it may be caused by acceleration of the gelation reaction of LAL with endotoxin in distilled water by heating or rise in pH before the assay [23].

Reproducibility of the gelation time for each endotoxin concentration in serum and rumen fluid was good with the ATK method. Coefficients of variation of endotoxin level were low and interassay variance of the gelation time was stable over a 12 days period.

Perchloric acid (PCA) pretreatment is now used in the chromogenic substrate method of endotoxin-specific chromogenic test [17]. Plasma protein containing interfering factors is removed by precipitation with PCA. However, the resulting precipitate also contains endotoxin activity [19], therefore the concentration of endotoxin may be higher than that indicated by this test result. Kambayashi et al. [7] also reported in plasma low concentration range the values obtained by the ATK method were higher than those obtained by chromogenic test. However, in the present ruminal high concentration range those obtained by ATK method were lower than those obtained by chromogenic test. The cause of this is not clear, though differences of protein and endotoxin volumes in rumen fluid and plasma may influence. Recently, Inada et al. [6] established a new PCA treatment method for removal of interfering factors, and in the future, original and revised methods should be compared.

The ATK assay method which is quantitatively accurate and simple, is useful for both experimental and clinical study of bovine endotoxin and detection of endotoxemia, and was therefore used in the study reported here.

Free endotoxin level in rumen fluid was related to the content of the ration. Ruminal endotoxin of steers fed Ration 4 was about ten times the level of those fed Ration 1, although there were great individual differences. Feeding of concentrate causes increase in ruminal histamine and lactic acid, while it results in decreased pH of rumen fluid and blood [2, 10]. Therefore, rumen function is greatly altered by concentrate as compared to basal ration 1, higher in roughage.

Changes in rumen function lead to a release of endotoxin from Gram-negative bacteria. Therefore, the rise in ruminal free endotoxin observed in cattle fed Ration 4 is likely explained. Although the present study detected significant differences in peripheral blood endotoxin level between cattle fed Ration 1 and Ration 4, all concentrations were extremely low. The explanation may be that endotoxin appears in the blood, only after it passes through the liver, where it is processed by the reticuloendothelial system [3, 22]. The amount of endotoxin reaching the peripheral blood is thus reduced.

Conversely, endotoxin concentrations in ruminal and other alimentary canal venous bloods may be influenced directly by free endotoxin generated in the rumen. It may be therefore become much higher than concentration in peripheral blood, since it reflects concentrations prior to passage through the liver. If this high concentration continuously reached the liver via the portal vein, it could cause hepatic injury [4, 18]. In this study, ruminal free endotoxin increased suddenly after Ration 1 was replaced with experimental rations with higher percentage of rolled barley. Rise in ruminal endotoxin was accompanied by a corresponding rise in blood endotoxin level. This sudden increase may be associated with the same changes which result in lactic acidosis [21].

During the time when rumen microflora have not yet adapted to the consumption of increased grain, release of endotoxin from dead Gram-negative bacteria likely increased. Yet, when the steers were fed Ration 4 continuously for two months, the ruminal endotoxin level was stabilized. This finding suggests that there is adaptation of rumen microflora to higher concentrate consumption after prolonged feeding.

In field observations, increase of endotoxin is observed in association with abrupt concentrate increase, when rumen microflora are not yet adjusted to ration change. At such time, grain engorgement diseases are most likely to occur. En-
detoxin levels detected in this experimental trial were in agreement with those expected, based on prior field experience.

ACKNOWLEDGEMENTS. We thank Dr. L. N. Brown, Veterinary Diagnostic Services, Rochester, Wash., U.S.A. and Dr. K. Tamura, Saikin Kagaku Institute Co., Sendai, for their helpful advices.

REFERENCES


