# Biological Activities of Lipopolysaccharides Extracted from Porcine Vaccine Strains

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ABSTRACT. Lipopolysaccharides (LPSs) were purified from *Actinobacillus pleuropneumoniae* serotype 2, *Bordetella bronchiseptica* and *Haemophilus parasuis* serotype 5, which were used for vaccine production in Japan, by the phenol-water procedure. In SDS-PAGE analysis, *A. pleuropneumoniae* LPS, as well as *Escherichia coli* LPS, demonstrated a typical ladder profile of a smooth-type LPS. On the other hand, *B. bronchiseptica* and *H. parasuis* LPSs lacked the ladder profiles. It was found that the biological activity of these LPSs was comparable to those of *E. coli* LPS in terms of activation of the clotting enzyme of *Limulus* amoebocyte lysate, mitogenic activity of mouse spleen cells, stimulation of TNF-α and nitric oxide production, but IL-6 production could hardly be observed in any LPS.—KEY WORDS: *Actinobacillus pleuropneumoniae*, biological activity, *Bordetella bronchiseptica*, *Haemophilus parasuis*, lipopolysaccharide.

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In domestic pigs, chronic infections which bring a reduction in feed efficiency, an increase in prophylactic medication and vaccination costs, and animal death, have recently been attracting more attention than acute epidemic infections. These chronic infections are mainly caused by gram-negative bacteria. Lipopolysaccharides (LPS), which are common components of the cell walls of gram-negative bacteria, are capable of eliciting a wide variety of pathophysiological effects, such as endotoxin shock, tissue injury, and lethality, in both humans and animals [13]. Inoculations of some domestic animals with gram-negative whole-cell vaccines are now increasing on the basis of epidemiological backgrounds. Accordingly, it seems important to clarify the effects of LPS not only on immunogenicity but also on toxicity so that a safety assessment of such vaccines can be made.

LPS is one of the most potent stimulators of monocyte/macrophage secretory response. *In vivo* and *in vitro*, monocytes/macrophages exposed to LPS induce a wide variety of pro-inflammatory mediators, such as tumor necrosis factor-alpha (TNF-α), Interleukin 6 (IL-6) and nitric oxide (NO) [4, 19, 22]. TNF-α is a most characterized cytokine that plays an important role in many inflammatory diseases and an apoptosis in the thymus [9]. In addition, it has become obvious that stimulation by bacterial LPSs are secreted NO. NO has also been shown to play a major role in the pathophysiology of septic and endotoxic shock [21].

*Actinobacillus pleuropneumoniae (A. pleuropneumoniae)*, *Bordetella bronchiseptica (B. bronchiseptica)* and *Haemophilus parasuis (H. parasuis)* are gram-negative bacteria which usually cause infectious disease in pigs [6,18, 20]. Bacterin or live vaccine has been widely used for the prophylaxis of these infectious diseases, but little is known about the biological activity of these bacterial LPSs with the exception of several reports on LPS extracted from *Escherichia coli (E. coli)*.

In this study, we examined the biological activities of LPSs from vaccine strains of *A. pleuropneumoniae*, *B. bronchiseptica* and *H. parasuis*, to obtain the underlying data for a safety assessment of veterinary vaccines.

## MATERIALS AND METHODS

*Bacteria and purification of LPS: A. pleuropneumoniae* serotype 2 strain SHP-1 and *H. parasuis* serotype 5 strain Nagasaki were obtained from the Nippon Institute for Biological Science, Tokyo, Japan. *B. bronchiseptica* strain S1 was obtained from the National Institute of Animal Health, Tsukuba, Ibaraki. These bacteria are used for vaccine production in Japan. *E. coli* O55:B5 LPS extracted by the hot phenol-water extraction method was purchased from Difco laboratories, U.S.A. Extraction of LPS from *A. pleuropneunomiae*, *B. bronchiseptica* and *H. parasuis* was done by the hot phenol-water extraction procedure of Westphal *et al.* [24], and purification with enzymes, which were 1 μg/m*l* of DNase I (Sigma, U.S.A.), 1 μg/m*l* of RNase A (Sigma), 1 μg/m*l* of trypsin 1:250 (Difco) and 1 μg/m*l* of Proteinase K (Wako Pure Chemical Industries Ltd., Japan), and ultracentrifugation (100,000 × g, 3 hr) were as described previously [2]. All LPSs were suspended in pyrogen-free saline or distilled water (Ohtsuka, Japan) at various concentrations.

*Detection of protein concentration in purified LPSs*: Protein concentrations in purified LPSs were measured by the Bio-Rad protein microassay procedure (Bio-Rad, U.S.A.) according to the manufacturer's instructions. The sensitivity of this microassay covers 1 to 20 μg protein. Bovine serum albumin (Sigma) was used as the standard protein. Results are shown as the mean of triplicate determinations ± standard error.

*Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*: Purified LPSs were subjected to SDS-PAGE analysis. The procedure involved the use of the Laemmli buffer system [10] and the separating and stacking gel of 12.5 and 4% acrylamide-bisacrylamide, respectively. After electrophoresis, the gels were stained

by the Tsai and Frasch silver staining procedure [23].

*Mitogenic assay*: The mitogenic activity of LPS was determined in BALB/c mice spleen cells [12]. Briefly, single-cell suspensions were cultured in RPMI 1640 medium supplemented with 1 mM L-glutamine, 10% fetal bovine serum, and 100 units/m*l* penicillin in microtiter plates to a final concentration of $3 \times 10^5$ cells/well. Triplicate cultures were incubated with or without LPS (20 ng/m*l* to 200 $\mu$g/m*l* in tenfold dilutions) at 37°C for 48 hr in 5% $CO_2$. After incubation, 25 $\mu l$ of MTT (5 mg/m*l* in PBS; Dojindo, Japan) was added to each well and incubated for 3 hr [8]. One hundred $\mu l$ of extract solution was added to each well and incubation was continued for a further 18 hr. Live cells were determined in an automated microplate reader MTP-32 (CORONA Electric Co., Japan) by measuring absorbance at 570 nm. Mitogenic assay was repeated three times.

*Limulus amoebocyte gelation assay*: Endotoxin levels were assayed by the kinetic turbidimetric method with the *Limulus* ES-II test (Wako Pure Chemical Industries Ltd.), an endotoxin-specific Limulus amoebocyte lysate (LAL-ES) test. Briefly, all LPSs were diluted 10-fold with pyrogen-free distilled water. A 0.1 m*l* sample of test fluid was added to 0.1 m*l* of LAL-ES in a glass tube. After vortex mixing for a few seconds, the gelation time was measured with a Toxinometer ET-201 (Wako Pure Chemical Industries Ltd.) and the specific activity was calculated with an LS-Toximaster (Wako Pure Chemical Industries Ltd.), a data acquisition program for the Toxinometer.

*Culture of RAW 264.7 cells and condition of LPS stimulation*: The mouse macrophage/monocyte cell line RAW 264.7 cells (ATCC TIB-71) were plated at $1 \times 10^6$ cells/2 m*l* in Dulbecco's modified Eagle's medium in microtiter plates and stimulated with 500 pg/m*l* to 5 $\mu$g/m*l* of several LPSs at 37°C in 5% $CO_2$. The culture media were collected at 24 hr for TNF-$\alpha$ and IL-6 bioassay and at 72 hr for NO assay.

*TNF-$\alpha$ bioassay*: TNF-$\alpha$ release in culture media was determined after 24 hr of incubation. TNF-$\alpha$ was measured by cytotoxicity assay on Wehi 164 clone 28–4 cells, in the presence of 1 $\mu$g/m*l* actinomycin D (Dojindo), as described previously [16]. Recombinant mouse TNF-$\alpha$ (Genzyme, U.S.A.) was used as a standard TNF-$\alpha$. Briefly, twofold dilutions of culture media were incubated with $5 \times 10^5$ cells/m*l* of Wehi 164 cells in RPMI 1640 medium in microtiter plates for 18 hr at 37°C in 5% $CO_2$. The cell viability was measured by MTT assay as described above. TNF-$\alpha$ bioassay was repeated three times.

*IL-6 bioassay*: IL-6 release in culture media was determined after 24 hr of incubation. IL-6 was measured by the assay on 7TD1 cells (ATCC CRL 1851). Recombinant mouse IL-6 (R & D Systems, USA) was used as a standard IL-6. Briefly, twofold dilutions of culture media were incubated with $2 \times 10^5$ cells/m*l* of 7TD1 cells in RPMI 1640 medium in microtiter plates for 48 hr at 37°C in 5% $CO_2$. The cell viability was measured by MTT assay as described above. IL-6 bioassay was repeated three times.

*NO assay*: NO activity was monitored as nitrite ($NO_2^-$) release in culture media after 72 hr of incubation, with Griess reagent [7]. Briefly, 100 $\mu l$ of Griess reagent was added to 100 $\mu l$ of diluted culture media in the wells of microtiter plates. After incubation at room temperature for 10 min, the absorbance at 570 nm was determined by means of an automated microplate reader MTP-32. NO assay was repeated two times.

*Lethal toxicity in D-galactosamine-treated BALB/c mice*: All LPSs were diluted in pyrogen-free saline and given intraperitoneally to BALB/c mice. To investigate the lethal toxicity of LPS, mice were sensitized by intraperitoneal injection of 20 mg of *D*-galactosamine (Wako Pure Chemical Ltd.)/mouse. Immediately after sensitization, the mice were intraperitoneally administered LPSs. Cumulative mortality was then monitored up to 24 hr. Fifty % of the lethal dose was calculated by the Reed & Müench method [17].

## RESULTS

*Purification of LPSs and SDS-PAGE profile*: Protein concentration in LPSs, purified from *A. pleuropneumoniae*, *B. bronchiseptica* and *H. parasuis* were 11.83 ± 0.34 $\mu$g/mg, 6.98 ± 0.22 $\mu$g/mg and 6.37 ± 0.43 $\mu$g/mg, respectively. The protein content in purified LPSs may be equated that of *E. coli* LPS (9.05 ± 0.41 $\mu$g/mg).

All LPSs had different patterns shown by SDS-PAGE analysis as shown in Fig. 1. *E. coli* and *A. pleuropneumoniae* LPSs demonstrated a typical ladder profile of a smooth-type LPS. On the other hand, *B. bronchiseptica* and *H. parasuis* LPSs were lacked the ladder profile and had a low molecular weight band detected at the leading edge of the dye front. In addition, *B. bronchiseptica* LPS had some broad bands, and *H. parasuis* LPS had two sharp bands.

*Mitogenic activity*: We desired to compare the mitogenic activity of *E. coli* LPS and purified LPSs according to MTT uptake by stimulated mice spleen cells (Fig. 2). *A. pleuropneumoniae*, *H. parasuis* and *E. coli* LPSs exhibited the activity but varied in their extent of activity. All LPSs showed that the activity at 20 $\mu$g/m*l* was higher than at other concentrations except for *B. bronchiseptica* LPS. Within the concentration range tested, such activities of *A. pleuropneumoniae* and *B. bronchiseptica* LPSs increased progressively, but those of *E. coli* and *H. parasuis* LPSs decreased at the 200 $\mu$g/m*l* concentration. All LPSs were therefore not clearly different from the proliferative activity from 20 *n*g/m*l* to 2 $\mu$g/m*l* by BALB/c mice spleen cells.

*Limulus amoebocyte gelation activity*: All LPSs activated the cascade of the clotting system of the horseshoe crab at a concentration of 10 *p*g/m*l*, but not at 1 *p*g/m*l* (data not shown). The specific activity of the LPSs, *A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis* and *E. coli*, were 4.7 EU/*n*g, 10.3 EU/*n*g, 5.6 EU/*n*g and 9.1 EU/*n*g, respectively.

*TNF-$\alpha$, IL-6 and NO production in culture media*: RAW 264.7 cells were stimulated for 24 hr or 72 hr with 500 *p*g/

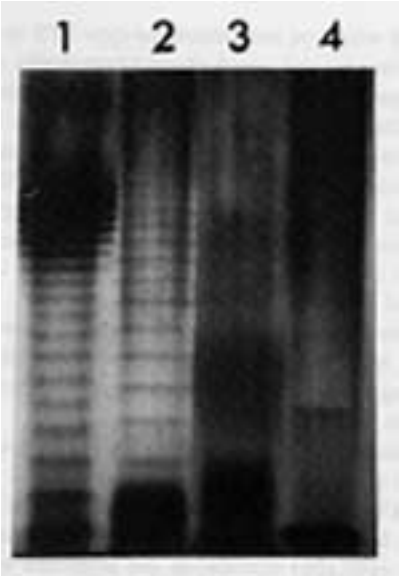


Fig. 1. Comparison of LPSs from *A. pleuropneumoniae, B. bronchiseptica* and *H. parasuis* and commercial *E. coli* LPS by SDS-PAGE with 12.5 % polyacrylamide gel. After electrophoresis, the gel was stained with silver. Lane 1, 10 $\mu$g of *E. coli* LPS; lane 2, 5 $\mu$g of *A. pleuropneumoniae* LPS; lane 3, 5 $\mu$g of *B. bronchiseptica* LPS; lane 4, 5 $\mu$g of *H. parasuis* LPS.

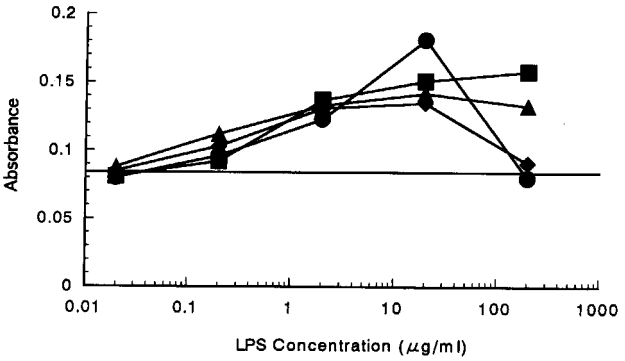


Fig. 2. Mitogenic activity of LPSs with MTT uptake by the BALB/c mice spleen cells. (●), *E. coli*; (▲), *A. pleuropneumoniae*; (■), *B. bronchiseptica*; (◆), *H. parasuis*. The line at 0.085 means the value obtained from BALB/c mice spleen cells as a control. Experiments were done three times, and representative results are shown. As standard errors were small, data are given as means.

m*l* to 5 $\mu$g/m*l* LPS. At the end of this time, the supernatants were collected for TNF-$\alpha$ and IL-6 quantitation.

Contrary to expectations there was no dose-dependent increase in the production of TNF-$\alpha$ by these cells after 24 hr LPS stimulation (Fig. 3a). The production of IL-6 could hardly be observed in any LPSs (Fig. 3b). In a comparison with *E. coli* LPS, we recognized that the dose-response curve of *E. coli* LPS was almost equivalent to that of the other three LPSs. On the other hand, there was a dose-dependent increase in the release of NO by these cells after 72 hr LPS stimulation (Fig. 4). Purified LPSs increased the release of NO at 5 *n*g/m*l*, but no NO activity was detected at the same dose of *E. coli* LPS.

*LPS-induced lethality in BALB/c mice*: The lethal toxicity of purified LPSs was tested in *D*-galactosamine-treated BALB/c mice. The 50% of lethal dose of *E. coli* LPS was calculated to be 13.4 *n*g/mouse, and purified LPSs, *A. pleuropneumoniae, B. bronchiseptica* and *H. parasuis*, were 7.8, 2.2 and 31 *n*g/mouse, respectively. *B. bronchiseptica* LPS was approximately one-sixth more active as to lethal toxicity in *D*-galactosamine-treated mice than *E. coli* LPS, but *H. parasuis* LPS was approximately a half less active than *E. coli* LPS.

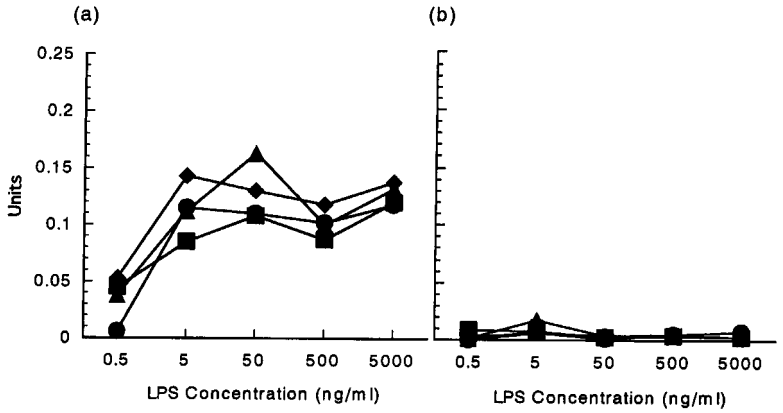


Fig. 3. TNF-$\alpha$ (a) and IL-6 (b) production from RAW 264.7 cells after 24 hr LPS stimulation. (●), *E. coli*; (▲), *A. pleuropneumoniae*; (■), *B. bronchiseptica*; (◆), *H. parasuis*. Experiments were done three times, and representative results are shown. As standard errors were small, data are given as means.

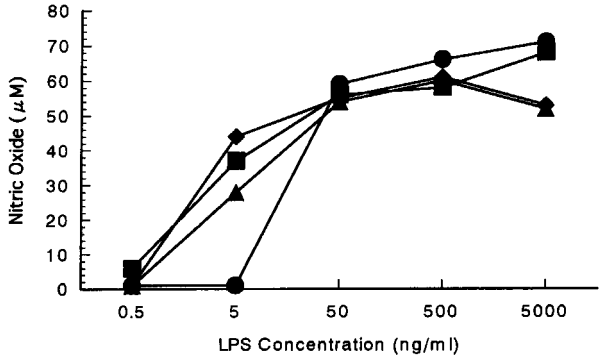


Fig. 4. Nitric oxide production from RAW 264.7 cells after 72 hr LPS stimulation. (●), *E. coli*; (▲), *A. pleuropneumoniae*; (■), *B. bronchiseptica*; (◆), *H. parasuis*. Experiments were done two times, and representative results are shown. Data are given as means.

## DISCUSSION

We examined the biological activity of the LPSs extracted from *A. pleuropneumoniae, B. bronchiseptica* and *H. parasuis*, which were used for vaccine production in Japan, in comparison with *E. coli* LPS as a control. Although some differences were observed among LPSs tested in a *Limulus* amoebocyte gelation activity and lethal toxicity in *D*-galactosamine-treated BALB/c mice, mitogenic activity and TNF-α, IL-6 and NO production were found to be similar, and were matched for that induced by the LPS of *E. coli*. The LPSs used in the present study included a small amount of protein. The possibility of its participation in the activity, however, seems to be small considering that the activity of LPS from *A. pleuropneumoniae*, of high protein content, was almost the same as that of LPS from *H. parasuis*, of low protein content. Furthermore, both LPSs were the same in *Limulus* gelation activity, which was caused entirely by LPS, not by protein.

The LPS structure of *A. pleuropneumoniae* serotype 2 extracted by phenol-water extraction was smooth-type LPS including a lipid A moiety [1, 14]. It was reported that only very small antigenic variations in the LPS components should also be observed in *B. bronchiseptica* [3]. In addition, it was shown that a classification of *B. bronchiseptica* isolates separated them into 6 immunogroups with reactivities with anti-*B. bronchiseptica* LPS monoclonal antibodies, and 6 porcine isolates were classified as immunogroups C2. Moreover, it was indicated that the LPS including immunogroups C2 contained both smooth- and rough-type LPS molecules as detected by tricine-SDS-PAGE analysis [11]. The smooth-type LPS in immunogroups C2 did not exhibited the ladder of sharp bands. As shown in Fig. 1, the *B. bronchiseptica* LPS extracted from the vaccine strain electrophoretically indicated both smooth- and rough-type LPS molecules on SDS-PAGE. It was also reported that *H. parasuis* LPS resembled wild-type enterobacterial-type LPS in non-ladder of sharp bands, and could electrophoretically distinguish the LPS profile of the vaccine strain from seven different LPS profiles in SDS-PAGE when stained with silver [25]. Our results showed that LPSs from the porcine vaccine strain used in this study had the characteristics of LPSs extracted from field isolates of each bacterium. Although the SDS-PAGE profile was different from all LPSs, the biological activities of LPSs were almost the same. It is therefore suggested that the polysaccharide structure, which has immunobiological activities, shows the difference among all LPSs, but lipid A structure, which plays a central role in the biological activity and toxicity of LPS, makes some difference.

The production of IL-6 was scarcely observed in any of the LPSs (Fig. 3b), but we demonstrated that $1 \times 10^6$ cells/m*l* of RAW 264.7 cells were stimulated with 50 μg/m*l* of *E. coli* LPS for 12 hr at 37°C in 5% $CO_2$. RNA was extracted from the cells after stimulation and amplified with specific IL-6 primers by the RT-PCR method. The specific IL-6 band could not be detected in any of the LPS non-stimulated cells, but was detected in LPS stimulated cells (data not shown). IL-6 mRNA was accumulated in RAW 264.7 cells by LPS stimulation, although scarcely any production of IL-6 was observed in the culture media. It has also been reported that lipid A, which is the active center of bacterial LPS, was induced into the production of IL-6 from RAW 264.7 cells [5].

We previously reported that apoptosis of lymphocytes was induced in lymphoid organs in pigs by *E. coli* LPS-injection and this suggests that LPS may affect the immune system in pigs [15]. It is believed that a depletion of lymphocytes in the lymphoid organs after an injection of gram-negative whole-cell vaccine may influence the prognosis by altering immunological functions. Further studies should be conducted to elucidate the immunological responses to LPSs extracted from vaccine strains in order to understand the effects of LPSs and to contribute to the assessment of the safety of veterinary vaccines to fight gram-negative bacterial infections.

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## REFERENCES

1. Altman, E., Brisson, J. R., Bundle, D. R. and Perry, M. B. 1987. Structural studies of the O-chain of the phenol-phase soluble lipopolysaccharide from *Haemophilus pleuropneumoniae* serotype 2. *Biochem. Cell Biol.* 65: 876–889.
2. Amano, K., Fukushi, K. and Watanabe, M. 1990. Biochemical and immunological comparison of lipopolysaccharides from Bordetella species. *J. Gen. Microbiol.* 136: 481–487.
3. Di Fabio, J. L., Caroff, M., Karibian, D., Richards, J. C. and Perry, M. B. 1992. Characterization of the common antigenic lipopolysaccharide O-chains produced by *Bordetella bron-*

*chiseptica* and *Bordetella parapertussis*. *FEMS Microbiol. Lett.* 97: 275–282.
4. Frolov, I., Houri-Hadad, Y., Soskolne, A. and Shapira, L. 1998. *In vivo* exposure to *Porphyromonas gingivalis* up-regulates nitric oxide but suppresses tumor necrosis factor-alpha production by cultured macrophages. *Immunology* 93: 323–328.
5. Funatogawa, K., Matsuura, M., Nakano M., Kiso M. and Hasegawa, A. 1998. Relationship of structure and biological activity of monosaccharide lipid A analogues to induction of nitric oxide production by murine macrophage RAW 264.7 cells. *Infect. Immun.* 66: 5792–5798.
6. Goodnow, R. A. 1980. Biology of *Bordetella bronchiseptica*. *Microbiol. Rev.* 44: 722–738.
7. Green, L. C., Wanger, D. A., Glogowsky, J., Sipper, P. L., Wishnok, J. S. and Tannenbaum, S. R. 1982. Analysis of nitrate, nitrite, and [$^{15}$N]nitrate in biological fluids. *Anal. Biochem.* 126: 131–138.
8. Hansen, M. B., Nielsen, S. E. and Berg, K. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/ cell kill. *J. Immunol. Methods* 119: 203–210.
9. Kato, Y., Morikawa, A., Sugiyama, T., Koide, N., Jiang, G.-Z., Takahashi, K. and Yokochi, T. 1995. Role of tumor necrosis factor-α and glucocorticoid on lipopolysaccharide (LPS) -induced apoptosis of thymocytes. *FEMS Immunol. Med. Microbiol.* 12: 195–204.
10. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage $T_4$. *Nature (Lond.)* 227: 6780–6785.
11. Le Blay, K., Gueirard, P., Guiso, N. and Chaby, R. 1997. Antigenic polymorphism of the lipopolysaccharides from human and animal isolates of *Bordetella bronchiseptica*. *Microbiology* 143: 1433–1441.
12. Moran, A. P., O'Malley, D. T., Vuopio-Varkila, J., Varkila, K., Pyhala, L., Saxen, H. and Helander, I. M. 1996. Biological characterization of *Campylobacter fetus* lipopolysaccharides. *FEMS Immunol. Med. Microbiol.* 15: 43–50.
13. Morrison, D. C. and J. L. Ryan. 1987. Endotoxins and disease mechanisms. *Annu. Rev. Med.* 38: 417–432.
14. Nakai, T., Horiguchi, Y. and Kume, K. 1990. Production and characterization of monoclonal antibodies against *Actinobacillus (Haemophilus) pleuropneumoniae* serotype 2. *Jpn. J. Vet. Sci.* 52: 533–542.
15. Norimatsu, M., Ono, T., Aoki, A., Ohisi, K., Takahashi, T., Watanabe, G., Taya, K., Sasamoto, S. and Tamura, Y. 1995. Lipopolysaccharide-induced apoptosis in swine lymphocytes *in vivo*. *Infect. Immun.* 63: 1122–1126.
16. Ogikubo, Y., Mori, Y., Kojima, A., Tamura, Y. and Yokomizo, Y. 1998. Production of recombinant porcine tumor necrosis factor alpha in a baculovirus expression system. *Jpn. J. Vet. Sci.* 60: 1203–1207.
17. Reed, L. J. and Müench, H. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hygene* 27: 493–496.
18. Sebunya, T. N. R. and Saunders, J. R. 1983. *Haemophilus pleuropneumoniae* infection in swine. A review. *J. Am. Vet. Med. Assoc.* 182: 1331–1337.
19. Shapira, L., Champagne, C., Van Dyke, T. E. and Amar, S. 1998. Strain-dependent activation of monocytes and inflammatory macrophages by lipopolysaccharide of *Porphyromonas gingivalis*. *Infect. Immun.* 66: 2736–2742.
20. Smart, N. L., Miniats O. P., Rosendal, S. and Friendship, R. M. 1989. Glasser's disease and prevalence of subclinical infection with *Haemophilus parasuis* in swine in southern Ontario. *Can. J. Vet.* 30: 339-343.
21. Szabo, C. 1995. Alterations in nitric oxide production in various forms of circulatory shock. *New Horizons* 3: 2–32.
22. Tonetti, M., Sturla, L., Giobinr, M., Benatti, U. and De Flora, A. 1995. Extracellular ATP enhances mRNA levels of nitric oxide synthase and TNF-α in lipopolysaccharide-treated RAW 264.7 murine macrophages. *Biochem. Biophys. Res. Commun.* 214: 125–130.
23. Tsai, C. M. and Frasch, C. E. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* 119: 115–119.
24. Westphal, O., Luderitz, O. and Bister, F. 1952. Uber die Extraktion von bacterien mit Phenol/Wasser. *Z. Naturforsch.* 7b: 148–155.
25. Zucker, B., Kruger, M., Rehak, E. and Horsch, F. 1994. The lipopolysaccharide structure of Haemophilus parasuis strains in SDS-PAGE. *Berl. Munch. Tierarztl. Wochenschr.* 107: 78–81.