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Brief Communication

Endotoxin-Effects of Vaccination with *Escherichia coli* Vaccines in the Pig

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Endotoxins from Gram-negative bacteria have been shown to be potent biological activators and are involved in many pathological reactions (*Yagoda et al.* 1990). Since endotoxins are ubiquitous compounds, they can create serious problems for the pharmaceutical industry. There are several examples of unavoidable drug contamination and of endotoxins that cannot be removed from recombinant procedures involving *Escherichia coli* bacteria (*Yagoda et al.* 1990). It is difficult to establish a true threshold dose at which endotoxins will cause clinical effects, but doses less than or equal to 3 ng/kg body weight (bw) can cause fever, chills and headache in humans (*Elin et al.* 1981). Another reference gives 5 endotoxin units (EU)/kg bw for similar clinical symptoms (*Anon.* 1987). Vaccines prepared using *E. coli* bacteria have a high potential for endotoxin content. This is important because endotoxin contamination in general can distort analyses of pharmacological studies and produce unpredictable and erratic effects on animals that receive contaminated vaccines. It is therefore important to identify possible effects of endotoxin-containing vaccines on various physiologic endpoints. It is also pertinent to assess the magnitude of any potential risks involved in using such vaccines. The purpose of this study was to evaluate the blood chemical and clinical response of castrated young boars to commercially available vaccines to *E. coli* where the bacteria have been formaldehyde killed and the endotoxins have not been removed.

Twelve castrated boars from 4 different litters with average weight 64.8 kg (range = 55-80 kg) were used in 2 identical experiments of 6 animals each. One additional pig (no. 13), weighing 71.5 kg, from the fourth litter was treated separately.

The vaccines used were Piliguard vet. (Batch no. 08145, Schering-Plough Corporation, Stockholm, Sweden) and Porcovac Plus vet. (Batch no. S118016/3, Hoechst, Stockholm, Sweden). They were analyzed for endotoxin content at Apoteksbolagets Centrallaboratorium, Stockolm, using the Limulus Amoebocyte Lysate (LAL) test according to Ph Eur V 219 Bacterial endotoxins. Both vaccines were found to have an endotoxin content between 75 000 and 125 000 EU/mL (Lysate sensitivity = 0.12 EU/mL). According to *Yagoda et al.* (1990), 100 000 EU corresponds roughly to 10 μg endotoxin calculated for *Salmonella typhimurium* endotoxin. The saline used for control animals contained <0.12 EU ml^{-1}.

Blood was sampled via a permanent catheter which was inserted in the jugular vein approxi-

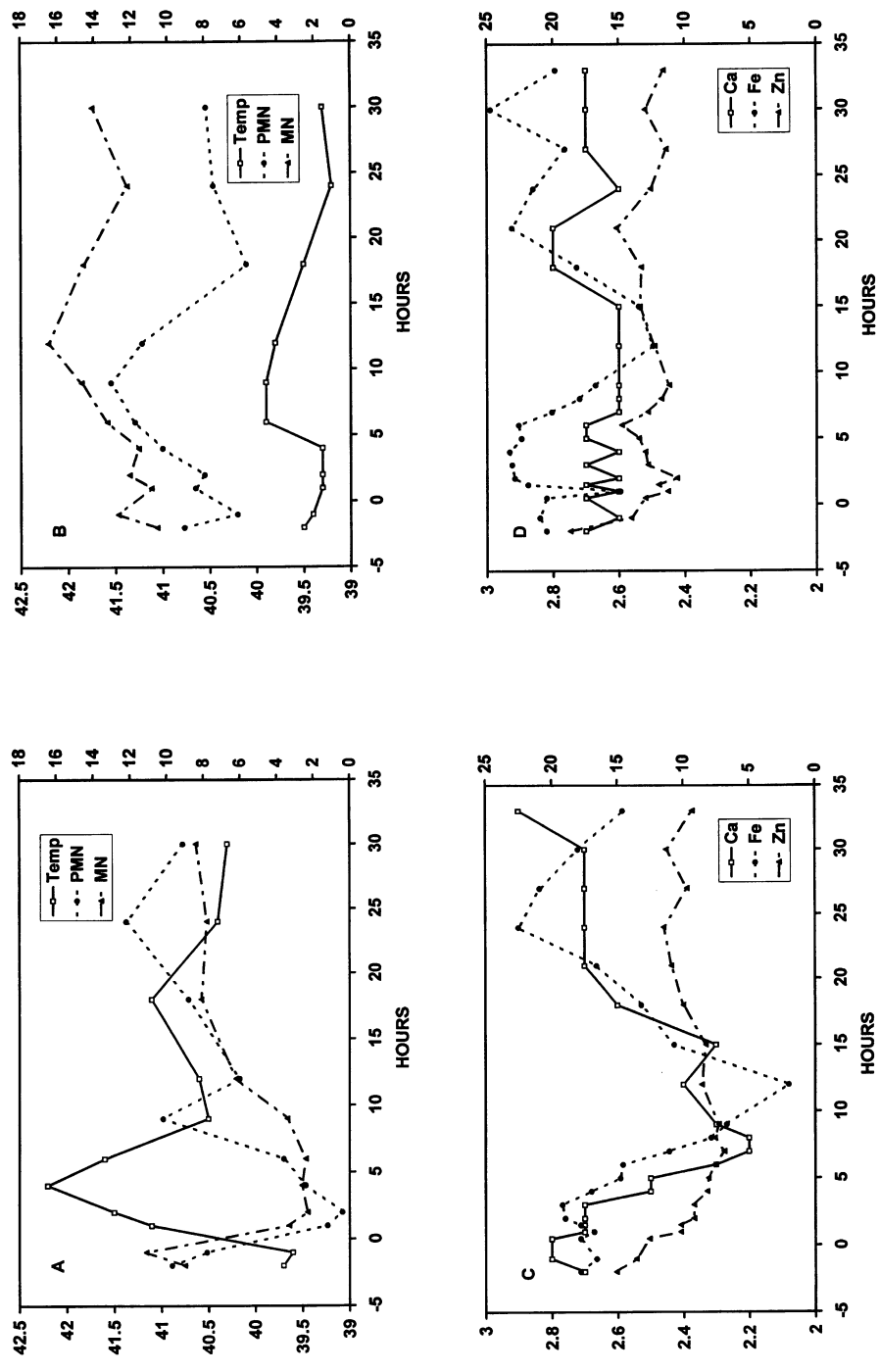
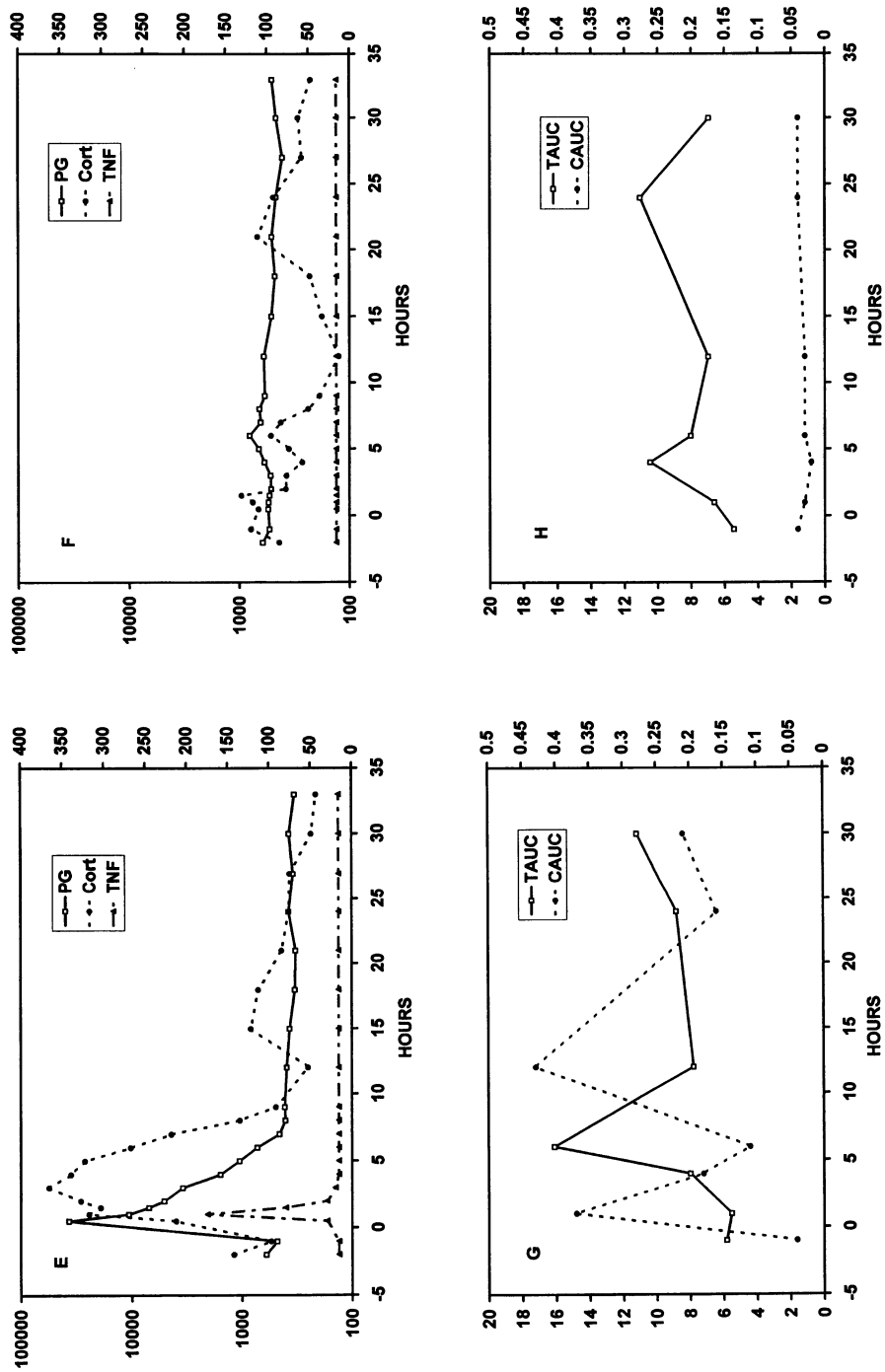


Figure 1. The responses after exposure of Piliguard vet. intravenously (pig no. 13, panels A, C, E and G) and strictly subcutaneously (pig no. 11, panels B, D, F and H).
Panels A and B: body temperature (Temp) in °C (left axis); and polymorphonuclear cells (PMN) and mononuclear cells (MN)$\times 10^9$ cells/L (right axis).
Panels C and D: calcium (Ca) in mmol/L (left axis); and iron (Fe) and zinc (Zn) in μmol/L (right axis).



Panels E and F: 15-ketodihydro-$PGF_{2\alpha}$ (PG) in pmol/L (left axis; note the logarithmic scale); and cortisol (Cort) in nmol/L and tumour necrosis factor (TNF) in pg/ml (right axis).
Panels G and H: phagocytosis-induced respiratory burst of the PMN:s (TAUC) (left axis); and spontaneous respiratory burst of the PMN:s (CAUC) (right axis). Both parameters are expressed as relative light unit per PMN in each blood sample.

mately one week before the experiment began, as described in *Rodriguez-Martinez & Kunavongkrit* (1983). Samples were collected in plain, heparinized, and EDTA- containing Vacutainer tubes, depending on the analyses to be performed.

In this experiment, 2 litters were used and one pig from each litter was randomly assigned to the following treatment groups: 1) control (2 mL saline), 2) Piliguard vet. (2 ml) or Porcovac Plus vet. (2 ml). Calculated on the endotoxin content, the injections of vaccines corresponded to about 3000 EU·kg^{-1} or 0.3 μg·kg^{-1} bw. The animals were restrained with a hog snare during the injections and all injections were given strictly subcutaneously. The thirteenth pig received 2 ml of Piliguard vet. intravenously and the resulting data was not included in the statistical analysis of the other 2 groups.

The injections were given at 0900 hours. Blood samples were collected in plain and heparinized tubes at 0700 and 0800 hours (before injection), at 0930, 1000, 1030, hourly until 1800 hours, and then every 3 hours until 1800 hours the following day. Blood was also collected in EDTA tubes and rectal temperature taken using an electronic thermometer at 0700, 0800, 1000, 1100, 1300, 1500, 1800, 2100, 0300, 0900, 1500 hours. Respiratory burst was assayed at 0800, 1000, 1300, 1500, 2100, 0900, 1500. Blood samples collected in plain tubes were allowed to clot at room temperature for 1 h before they were centrifuged and serum was pipetted off and stored at –20 °C for later analysis. The heparinized blood was either centrifuged and separated immediately (plasma stored at –20 °C until analysis) or was used for measuring the granulocytes' spontaneous or phagocytosis-induced respiratory burst according to *Magnusson & Holst* (1998) and then centrifuged. Blood in EDTA tubes was analyzed for leukocyte counts and smears were made immediately after collection except for those collected at 2100 and 0300 hours. Those samples were smeared immediately but kept refrigerated until the next morning when the rest of the analyses were done. The reason for leaving the 2100 and 0300 hours samples until next morning was practical, but the groups were treated in the same way.

Serum-Fe levels were measured using reagents from Roche Diagnostica, Basel, Switzerland, according to the method described in *Eisenwiener et al.* (1979). Serum-Ca and S-Zn levels were measured using an atomic absorption spectrophotometer according to *Trudeau & Freier* (1967) and *Parker et al.* (1967), respectively. Total WBC count was done using an automatic cell counter and differential polymorphonuclear/mononuclear (PMN/MN) counts were obtained by microscopic counting of 100 cells from May-Grünwald/Giemsa stained smears. The resulting percentages were multiplied by the total WBC count to yield absolute values.

Analysis of 15-ketodihydro-$PGF_{2\alpha}$ was done using the radioimmunoassay method as described in *Granström & Kindahl* (1982). Plasma-Cortisol levels were measured by a competitive radioimmunoassay according to *Österlundh et al.* (1997). The content of tumour necrosis factor (TNF-α) was determined in the plasma of pigs no. 11 and no. 13 (TNF-α, Predicta Elisa kit, Genzyme, Cambridge, MA, USA). The detection limit was 16 pg/mL.

The effect of the various treatments was analyzed by a general linear model (SAS). The random effect of animal nested within treatment regime was used as an error term when testing treatment effects.

In the main experiment involving 12 animals nos. 1, 4, 7 and 10 received saline; nos. 2, 5, 8 and 11 received Piliguard vet.; and nos. 3, 6, 9 and 12 received Porcovac Plus vet. There were no clinical or blood biochemical differences between the treatments in body temperature, S-Ca, S-Fe, S-Zn, total WBC + differential

counts, prostaglandin metabolite and cortisol levels or granulocytes' respiratory burst.

The 13th pig, which received the vaccine intravenously, showed changes in all the studied parameters. This animal is depicted in Fig. 1 and as a comparison its littermate pig no. 11 is shown. The latter pig received the same type of vaccine as no. 13, but it was administered subcutaneously. Boar no. 13 showed all the classic symptoms of endotoxaemia. It was depressed and lying down for 2-5 h after the injection. No studied compounds except endotoxins can elicit such a response in prostaglandin metabolite levels (>36 $nmol \cdot l^{-1}$ plasma) as was demonstrated in this animal. Also the low numbers of PMN:s seen 2 h after the injection of vaccine (4.6% of pretreatment levels) clearly indicate a strong exposure to endotoxins. Interestingly the intravenous injection seemed to strongly increase the granulocytes' spontaneous respiratory burst. No changes were seen in the phagocytosis-induced respiratory burst. The pronounced rise in cortisol levels also indicates an exposure to endotoxins (*Magnusson et al.* 1994). The decrease in Ca, Zn and Fe is also a clear indication of endotoxin exposure (*Holst et al.* 1993). This boar also shows a clear and distinct peak in the plasma concentration of TNF, which is in accordance with studies in which endotoxin has been administered intravenously to pigs (*VanderMeer et al.* 1994). No elevation in the TNF values was seen in pig no. 11. In the rough estimate of endotoxin amounts, pig no. 13 should have received about 0.3 $\mu g \cdot kg^{-1}$ bw (or 3000 EU/kg bw). Earlier studies in the pig have used somewhat higher doses of *Salmonella typhimurium* endotoxin, 0.5-3 $\mu g \cdot kg^{-1}$ bw, but *Cort* (1986) and *Cort et al.* (1986) found small differences among these doses in, for example, prostaglandin release and efficacy of inducing abortions, and concluded that the high doses were excessive for inducing these effects. It is somewhat surprising that the animals that received the vaccine strictly subcutaneously did not show any clinical or blood biochemical changes as compared to the large changes seen in pig no. 13, which received the same dose intravenously. Obviously the uptake from the subcutaneous depot is slow and the endotoxins cannot create the same negative effects when given subcutaneously as they can when administered intravenously. Under clinical field circumstances the vaccinations are performed subcutaneously/intramuscularly and the uptake from the injection site can vary. However, there is a risk of the vaccine coming directly into the circulation through small blood vessels.

The castrated boars received the same dose of the vaccines as recommended for pregnant gilts or sows in late pregnancy. The recommendations are slightly different for the 2 vaccines used, but in general the first vaccination should be done 6 weeks before expected farrowing and the second 2-4 weeks later or equal to 2-4 weeks before expected farrowing. Thus the first vaccination is done approximately on day 70 of pregnancy. Re-vaccination is recommended about 2-4 weeks before expected farrowing or at 85-100 days of pregnancy. Pregnant gilts have shown to be vulnerable to endotoxins and abort in this period of pregnancy (*Cort* 1986, *Cort et al.* 1986). Later on in pregnancy (between 80 and 100 days) the abortifacient effect of endotoxins is decreased but fetuses might die in utero (*Cort & Kindahl* 1986). Pregnant gilts in this gestational period have a body weight at least the double of boar no. 13, but as mentioned above the abortifacient effect of endotoxins is not dose-dependent. Thus pregnant animals in a susceptible period of pregnancy receive vaccines with a substantial amount of endotoxins. It is not possible to demonstrate any negative effects after the recommended subcutaneous injections, but the same dose of vaccine intravenously can cause a severe endotoxaemia with a potential risk of abortions.

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References

Anonymous: FDA's Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices, 1987.

Cort N: A clinical study on the effects of a Gram-negative bacterial endotoxin and cloprostenol in non-pregnant and 60 days pregnant gilts. Anim. Reprod. Sci. 1986, *10,* 133-145.

Cort N, Kindahl H: The effect of a bacterial endotoxin or cloprostenol on the clinical status and hormonal levels in 80-100 days pregnant gilts. Acta vet. scand. 1986, *27,* 145-158.

Cort N, Kindahl H, Einarsson S: The effect of a Gram-negative bacterial endotoxin and cloprostenol on the plasma levels of 15-keto-13,14-dihydro-$PGF_{2\alpha}$, progesterone, oestradiol-17β, oestrone sulphate and luteinizing hormone in non-pregnant and 60 days pregnant gilts. Anim. Reprod. Sci. 1986, *10,* 147-162.

Eisenwiener H-G, Rietz P, Schläpfer P: Die Bestimmung des Eisens mit der Guanidiniumchlorid/Ferrozin-Methode. J. Clin. Chem. Clin. Biochem. 1979, *17,* 149.

Elin RJ, Wolff SM, McAdam KPWJ, Chedid L, Audibert L, Bernard C, Oberling F: Properties of reference *Escherichia coli* endotoxin and its phthalylated derivative in humans. J. inf. Dis. 1981, *144,* 329-336.

Holst H, Edqvist L-E, Kindahl H: Reduced response to intravenous endotoxin injections following repeated oral administration of endotoxin in the pig. Acta vet. scand. 1993, *34,* 405- 419.

Granström E, Kindahl H: Radioimmunoassay of the major plasma metabolite of $PGF_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$. Methods Enzymol. 1982, *86,* 320-339.

Magnusson U, Holst H, Kindahl H, Karlsson Å: Effect of mimicking prepartum concentrations of estradiol-17β on the inflammatory response to endotoxin in gilts. Am. J. Vet. Res. 1994, *55,* 785-789.

Magnusson U, Holst H: Assaying granulocyte phagocytosis in vitro: effects of storage time and temperature of blood samples. J. Vet. Med. B 1998 (in press).

Parker MM, Humoller FL, Mahler DJ: Determination of copper and zinc in biological material. Clin. Chem. 1967, *13,* 40-48.

Rodriguez H, Kunavongkrit A: Chronical venous catheterization for frequent blood sampling in unrestrained pigs. Acta vet. scand.1983, *24,* 318-320.

Trudeau DL, Freier EF: Determination of calcium in urine and serum by atomic absorption spectrophotometry (AAS). Clin. Chem. 1967, *13,* 101-114.

VanderMeer TJ, Menconi MJ, O'Sullivan BP, Larkin VA, Wang H, Kradin RL, Fink MP: Bactericidal/permeability-increasing protein ameliorates acute lung injury in porcine endotoxemia. J. Appl. Physiol. 1994, *76,* 2006-2014.

Yagoda CR, Bylund-Fellenius A-C, Adner N, Kindahl H: Biological responses of sheep treated with endotoxin-contaminated superoxide dismutase and endotoxin preparations. Acta vet. scand. 1990, *31,* 207-217.

Österlundh I, Holst H, Magnusson U: Hormonal and immunological changes in the mammary gland of the sow at parturition. (Submitted for publication).

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