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Dietary specific antibodies in spray-dried immune plasma prevent enterotoxigenic
Escherichia coli F4 (ETEC) post weaning diarrhoea in piglets.

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Abstract

In order to establish the mechanism of spray dried plasma powder (SDPP) in improving pig health and performance, a diet containing either 8% SDPP, spray dried immune plasma powder (SDIPP), or control protein (soybean and whey) ration was fed to piglets in an experimental model of enterotoxigenic *Escherichia coli* F4 (ETEC) post-weaning diarrhoea (PWD). SDIPP was obtained from pigs immunized with a vaccine containing ETEC fimbrial subunit F4 and heat-labile toxin (LT), and SDPP from non-immunized controls. Average daily growth (ADG) was determined, and daily samples of rectal faeces were assessed for diarrhoea (as percentage of dry matter), and ETEC excretion (in CFU/g).

SDPP and SDIPP ($p < 0.05$) significantly reduced diarrhoea, and SDIPP significantly reduced ETEC excretion. ADG was not significantly ($p > 0.05$) affected. After the experiment, 30% of piglets tested F4 receptor positive (F4R+). A significant correlation between F4R status and morbidity was found. In F4R+ animals, SDIPP significantly improved diarrhoea and ADG, and decreased ETEC excretion, and SDPP significantly improved diarrhoea and ADG. Surprisingly, SDPP reduced diarrhoea in F4R+ animals without significant reduction of ETEC excretion, which is most likely related to the presence of anti-LT antibodies in SDPP.

The results show that oral protection against ETEC by SDPP is attributable to spontaneous antibodies, in this case anti-LT antibodies. Furthermore, the results indicate that the combination of anti-LT and anti-F4 antibodies as in SDIPP is most effective in ETEC prevention. Finally, the F4R distribution in the herd should be taken into account to correctly assess efficacy.

1. Introduction

Spray dried plasma powder (SDPP) is a common protein source in starter diets for weaned piglets in many countries. It is described to improve growth and health during the first days after weaning, in particular under high infection pressure (Coffey and Cromwell, 1995, Bergstrom et al., 1997). Results are varying, which is probably also related to the susceptibility for one of the most common diseases in the post-weaning period, post weaning diarrhoea (PWD). Enterotoxigenic *Escherichia coli* (ETEC) induced PWD is an important cause of morbidity and mortality in neonatal and weaned piglets. In pigs, successful intestinal colonization of ETEC strains is associated with bacterial adhesion factors (fimbriae), such as F4 (Gaastra and de Graaf, 1982). Upon colonization ETEC can produce several toxins among which heat labile toxin (LT), which cause secretory diarrhoea. Immunization of the sow with ETEC adhesion factors and LT protects suckling piglets against PWD, weaning withdraws lactogenic protection, and maternal colostrum-derived systemic antibodies in piglets are ineffective (Francis and Willgoos, 1991). Dietary inclusion of specific antibodies raised against F4 (Yokoyama et al 1992, de Geus et al., 1998, Owusu-Asiedu et al., 2002, 2003) protected against *E. coli* F4 ETEC. The mechanism behind SDPP is unclear, transfer of passive immunity has been proposed (van Dijk et al., 2001), but only in one case specific anti-F4 antibodies were found in SDPP (Owusu-Asiedu et al., 2002).

The objectives of the present study were 1. to establish the effect of addition of SDPP and spray dried immune plasma (SDIPP) to the weaning diet on prevention of enterotoxigenic *E. coli* F4 post-weaning diarrhoea (PWD), 2. to get insight into the role of specific antibodies in the pathogenesis of ETEC, and 3. to determine the influence of the F4R status on this process.

2. Material and methods

2.1. Bacterial strains

E. coli O149K91 was isolated from a pig farm with post weaning diarrhoea. The ETEC strain was typed at the Animal Sciences Group in Lelystad, and further characterized as F4 (K88ac), LT+, STb+ (Nabuurs et al., 1993), and designated CVI-1000. As a control in the brush border adhesion studies CVI-1084 was used, a strain identical to CVI-1000 but without F4 fimbrial expression (a gift from Dr. F. van Zijderveld, Department of Bacteriology, Animal Sciences Group, Lelystad, the Netherlands). The strains were grown overnight in brain heart infusion broth (Difco Laboratories, Detroit, MI, USA), pelleted by centrifugation and resuspended in PBS (pH 7.2) to an extinction at 600 nm, corresponding to 10⁹ CFU/ml.

2.2. Post weaning diarrhoea (PWD) animal model

The protocol was approved by the local Ethics Committee for Animal Experiments in Lelystad. PWD is induced in piglets weaned at 3 weeks of age. The induction protocol involves cold stress, fasting, treatment with colistin to disturb intestinal ecology, inoculation with rotavirus, and inoculation with ETEC. Animals (96 from 16 different litters, 3 wk of age) were purchased from a commercial piggery, and transported to our facilities on the day of weaning. Piglets were housed in 4 multipurpose boxes, each containing 3 pens, separated by closed planking, containing 8 piglets each. Piglets were divided over the pens, taking litter, weight, and sex into account. Piglets were kept at a 16 h light, 8 h dark cycle, at 24°C. Animals were fasted on day 1 and 2. Water was given ad lib throughout the experiment. Colistin sulphate (Eurovet, Bladel, The Netherlands)(60 mg/L) was added to the drinking

water on the first 5 days. From day three on, animals had ad lib access to one of the three diets (containing SDPP, SDIPP, or control protein). On day 5, all piglets were orally inoculated by syringe with 2 ml (10^6 particles/ml) rotavirus strain RV277 (Debouck and Pensaert, 1979) suspension. On day 6 and 7, all animals received 5 ml ETEC suspension of 10^9 CFU/ml. From day 2 on, each day the animals were weighed, their health was assessed, the total food intake per pen was determined for determination of average daily food intake (ADFI) and feed conversion ratio (FCR). Daily, a rectal content sample was taken for determination of the % dry matter (%DM), and for determination of ETEC counts. On day 15, the animals were euthanized by Nembutal, bled, and heparin plasma was taken. Gross pathology was performed, and a jejunal sample was taken for determination of F4R status by brush border adhesion assay. On animals found dead gross pathology was performed and, if possible, a plasma and a jejunal sample were collected.

2.3. Determination of the percentage dry matter (%DM) of faeces

Faeces (0.5-3 g) was weighed into aluminium trays. Samples were desiccated for 20 h in an incubator at 80 °C, and weighed again to determine lost water.

2.4. Quantification of ETEC excretion

One gram of faeces was weighed into 10 ml tubes, and 9 ml of 0.9% NaCl was added, and vortexed. Dilutions were plated on sheep blood agar plates containing 5% sheep blood, streptomycin 50 µg/ml, tetracycline 25 µg/ml, vancomycin 50 µg/ml (Biotrading, The Netherlands). Haemolytic colonies with the typical appearance of *E. coli* were counted. In cases of ambiguous colony morphology, identity was confirmed by slide agglutination.

2.5. Source of plasma, and immune plasma powder

At a large commercial breeder and fattener, animals (n=320) were immunized (i.m. in the neck) with 2 ml of Porcilis coli (Intervet International BV, Boxmeer, The Netherlands) containing adhesion factors K88ab (F4), K88ac (F4), K99 (F5), 987P (F6), and heat labile toxin (LT). Pigs were immunized at 6, 4, and 2 weeks before slaughter (at ca. 115 kg live weight). The non-immunized control group (n= 320) was from the same farm, and from the same period. At the slaughterhouse of the farm, blood was collected separately per animal into citrated bags. After post mortem inspection, blood was pooled per group (ca 1000 L), transported to the plasma protein factory (Sonac), plasma was separated by centrifugation, the pH increased to 10 using a 25% ammonia solution, and stored overnight at 2°C. The next day, plasma was two-fold concentrated by ultra filtration (membrane cut-off 9 kD, MSD3 Ultra filtration unit, Stork, The Netherlands), and spray dried in an Anhydro Lab S1 spray dryer (Anhydro, Copenhagen, Denmark), at an outlet temperature of 80-83 °C. Since antibiotics possess growth promoting and antibacterial activity, anti-microbial drug residue was determined by the well diffusion method, using 10% SDPP and SDIPP in PBS in 9 mm diameter wells in 5 different 2 mm agar plates seeded with *Micrococcus luteus* at pH 6 or pH 8, and *Bacillus subtilis* at pH 6 or pH 8 with or without Trimethoprim. After overnight incubation at 37°C the inhibition zone was measured. An inhibition zone < 3 mm is considered to be negative. Microbiological quality was determined according to the International Standard, for total bacterial count (ISO 4833:1991(E), for *Enterobacteriaceae* (ISO 7402:1993(E), and *Salmonella spp* (ISO 6579:1993(E). The percentage dry matter was determined by drying at 103 °C, and the soluble fraction in water was determined gravimetrically.

2.6. Production of pelleted feed

The composition of the three experimental diets is shown in Table 1. The diets contained either soybean (meal) and whey powder, or SDPP, or SDIPP as main protein source. The diets were pelleted at 61 °C, 66 °C and 67 °C, respectively and were formulated to contain 1.03 % apparent ileal digestible lysine, 2352 kcal NE/kg and 4.7 % lactose (van Dijk et al., 2002). Analysis of feed was performed as described before (van Dijk et al., 2002). The total immunoglobulin content of SDPP, SDIPP, whey powder and the diets was determined by protein-G affinity chromatography with UV detection (Pharmacia/LKB, Uppsala, Sweden).

2.7. Immunological analysis

Pelleted samples were first ground up to powder. Dry powder samples were incubated overnight 10% w/v in PBS at 4°C. Samples were then centrifuged and the supernatant analyzed. Samples were analyzed by ELISA for the presence of specific antibodies. Pooled plasma from specific pathogen free (SPF) piglets from the Lelystad facilities served as negative control. Indirect ELISA for determination of anti-F4 and anti-LT titre was performed according to van Zijderveld et al. (1990), and Lauterslager et al. (2001) respectively, using 96-wells microtiter plates coated with 0.5 µg purified F4ac/well or 0.05 µg LT (Sigma, St. Louis, Missouri, USA)/well. Samples were serially diluted, incubated, washed, and bound antibodies were detected with HRPO-labelled rabbit anti-swine immunoglobulins (Dakopatts, Copenhagen, Denmark). Titres were expressed as EC50.

2.8. Determination of piglet F4R status by brush border adhesion (BBA) assay

At necropsy, 5-10 cm of jejunal mucosa was scraped off, and epithelial brush borders were prepared, and the F4R status determined using the BBA method modified after Sellwood et al. (1975). The number of bacteria attached to 50-100 cells with well-defined brush borders were counted by phase contrast microscopy (magnification, x 400). Animals with no or an average of 1-2 bacteria/brush border were considered F4R-, samples exceeding this are judged F4R+. In case of ambiguity, the test was repeated.

2.9. Brush border adhesion (BBA) inhibition assay

Functionality of plasma was determined by the BBA assay described above, modified as described (Harmsen et al., 2005). Briefly, serial dilutions of plasma preparations were added to brush border preparations. Then the bacterial suspension was added, and bacterial adhesion was determined. Titres were expressed as the reciprocal highest dilution showing complete inhibition of BBA.

2.10. LT Vero-cell assay

Functionality of anti-LT antibodies was assayed in Vero cells (Speirs et al., 1977). Pooled plasma from SPF-piglets from the Lelystad facilities served as negative control.

2.11. Statistics

The piglet was the experimental unit. A four factorial analysis on the effects of the different feeds, the two F4 receptor types, box and pen was carried out. There were no main

and interaction effects of box and pen and consequently these factors were omitted from further analysis. A two factorial ANOVA with the factors FEED (control, SDPP, SDIPP) and F4Receptor (F4R+, F4R-) was performed (GLM procedure, SAS version 9.1, SAS Institute, Cary, NC). In case of significant FEED by F4R interactions one factorial ANOVA was performed with the factor Group (representing all 6 combinations of FEED and F4R) supplemented by Fisher's least significant difference (LSD) post hoc comparisons. Statistical significance was predefined at $p < 0.05$. Data are presented as mean \pm sem.

3. Results

3.1. Feed analysis

Analysis of pelleted feed showed that the analyzed values for feed composition were in line with those calculated (Table 1). The immunoglobulin content of control feed was below the detection limit, in undiluted preparations the immunoglobulin content was 1.3 % in whey powder, 23.7 % in SDPP, and 25.7 % in SDIPP.

3.2. Production of porcine plasma powder, analysis of SDPP and SDIPP, and survival of antibody titres in the production process

From 320 animals, a little over 20 kg spray dried plasma powder was obtained (Table 2a). Microbial quality was within the industrial norm. Antimicrobial drug residue activity was below the detection limit (Table 2b). Immune plasma did shown high anti-F4 titres, whereas normal plasma did not. Immune plasma showed BBAI reciprocal titre of >100 , normal plasma, and SPF plasma <3 . Survival of anti-F4 titres in SDIPP was 70% after spray drying,

no further reduction was found at pelleting (Table 2c). Further testing for anti-LT immunoreactivity showed that immune plasma had a titre of 976 ± 241 (n=2), and normal plasma 140 ± 25 (n=2). In the Vero cell bio-assay, at a dilution of 1:50, both normal plasma and immune plasma completely inhibited LT cytotoxicity, whereas SPF plasma did not.

3.3. The effect of diet composition on PWD ignoring the F4R status

Five animals out of 96 (5.2 %) were found dead during the experiment. One animal died at day 5, due to hernia diafragmatica. The other 4 deaths were associated with PWD, and were found in the control group (1) at day 8, 13, 14, and one in the SDIPP group at day 12. Weight of the animals at the start of the experiment was 8.04 ± 0.16 kg (n = 91), and no significant differences were found concerning box or diet. Faecal %DM of all three diets was 29.0 ± 0.8 pre-infection (day 1-6). ADG did not significantly differ between diets on days 1-6. After inoculation with ETEC, control animals (n = 29) excreted high numbers of ETEC (Fig 1). Feeding SDPP (n = 32) did lower ETEC excretion, but not significantly. SDIPP (n = 30) did lower faecal ETEC counts compared to both control and SDPP. Concerning %DM, the lowest % was found in the control group. Feeding SDPP improved this significantly, and SDIPP improved it even further, being significantly different from both control and the SDPP group. Concerning ADG no significant differences ($p > 0.05$) were found between the diets, nor for ADFI (0.278 ± 0.023 kg/day in the control group, increasing to 0.316 kg/day in SDPP, and 0.326 kg/day in SDIPP). FCR did not differ between diets during the entire experiment (results not shown).

3.4. The effect of diet composition on PWD taking the F4R status into account

In the brush border adhesion assay, 30% tested F4R+, no ambiguous results were seen. The F4R distribution varied between the different treatments, F4R+ vs F4R- was in the control group 10 vs 19, in the SDPP group 10 vs 22, and in the SDIPP group 8 vs 22. There was a significant interaction of FEED x F4R. No significant differences between F4R- animals were found in all three treatments. In the control animals, F4R status dependent significant differences were found for all three parameters, F4R- animals excreted less *E. coli*, had a higher faecal %DM, and a higher ADG. Concerning *E. coli* excretion, feeding SDPP did not have a significant effect on the difference between F4R+ and F4R- animals. Feeding SDIPP significantly lowered *E. coli* excretion in F4R+ animals compared to F4R+ animals in both the control and the SDPP group. Concerning faecal %DM, in F4R- animals, feeding SDPP gave a significant improvement compared to controls, and SDIPP was significantly better than both the control and SDPP. Both SDPP and SDIPP significantly improved ADG in F4R+ animals compared to the controls, no significant difference was found between SDPP and SDIPP. ADFI and FCR did not differ between diets during the entire experiment (results not shown).

3.5. Anti-F4 and anti-LT antibodies in piglet plasma

Plasma of all piglets contained antibodies to both F4 and LT, in varying titres. No significant relationship was found with diet, box, pen, litter, *E. coli* excretion, faecal %DM, ADFI, FCR or F4R status (results not shown), consistent with maternal colostrum derived antibodies.

Discussion

In the present study, we set out to determine whether SDPP has indeed beneficial properties, with particular attention to the role of specific antibodies. We have used a relatively cheap and readily available source of SDPP, and compared it with immune plasma SDIPP, in our model of post-weaning diarrhoea (PWD). Both SDPP and SDIPP were included in pelleted feed in order to work as realistic as possible. Processing may seriously affect bioactivity. As judged by anti-F4 titre in SDIPP, the most critical step in the production process is spray-drying at which not more than 30 % of the activity is lost. Further processing to pellets does not lead to appreciable losses.

We have demonstrated that both SDPP and SDIPP do improve performance and health, similar to what has been shown in neonatal diarrhoea using egg yolk or SDPP derived antibodies (Yokoyama et al., 1992, Owusu-Asiedu et al., 2002). We also show that this happens in a F4R status dependent way. SDIPP protected against PWD, and this could be attributed to the presence of both anti-LT and anti-F4 antibodies. SDPP protected only significantly against diarrhea, which is consistent with the presence of anti-LT antibodies only. The unanticipated presence of anti-LT antibodies in SDPP probably reflects natural exposure to pathogens, similar to what has been found in normal bovine colostrum (Rump et al., 1992). The absence of anti-F4 antibodies is consistent with the absence of significant colonization inhibition by SDPP as is particularly evident in F4R+ piglets. Our SDPP did not contain anti-F4 activity as opposed to the commercial preparation used by Owusu-Asiedu et al., 2002. It is reasonable to assume that batch differences in natural antibodies is one of the possible causes for variation in results obtained with SDPP. Another source of variation could be the differences in prevalence of F4R-status of the herds used in the various experiments. In most studies, piglet F4R status is not mentioned. Here we found that *E. coli* F4 can maintain itself at relatively high levels (up to 10^5 /g faeces on a given day) in F4R- animals in an apparent F4R independent manner. This level is affected by the presence of sufficient number

of F4R+ animals which maintain a high infection pressure by excreting high levels of bacteria (Geenen et al., 2005).

Our findings confirm that SDPP reduces PWD because it contains specific antibodies, in our case anti-LT antibodies. Comparing SDPP and SDIPP it is evident that the latter not only protects against diarrhoea, but also reduces ETEC excretion, which will reduce transmission within a herd (Geenen et al., 2005). Finally, these positive results were obtained in a herd in which a relatively low percentage (30%) of pigs was F4R+. It is clear that a much larger effect can be expected in higher F4R+ prevalence herds.

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374

375 Figure 1. The effect of SDPP and SDIPP on ETEC excretion, (in log CFU/g faeces),
376 diarrhoea as % DM, and performance (ADG). Left panel with shaded bars represent results
377 obtained without taking the F4R status into account. In the right panel, the results per
378 treatment for F4R+ (open bars), and F4R- (black bars), respectively are shown.
379 Data are mean \pm s.e.m. Significant differences ($p < 0.05$) between groups/treatments are
380 indicated with lines and asterisks.

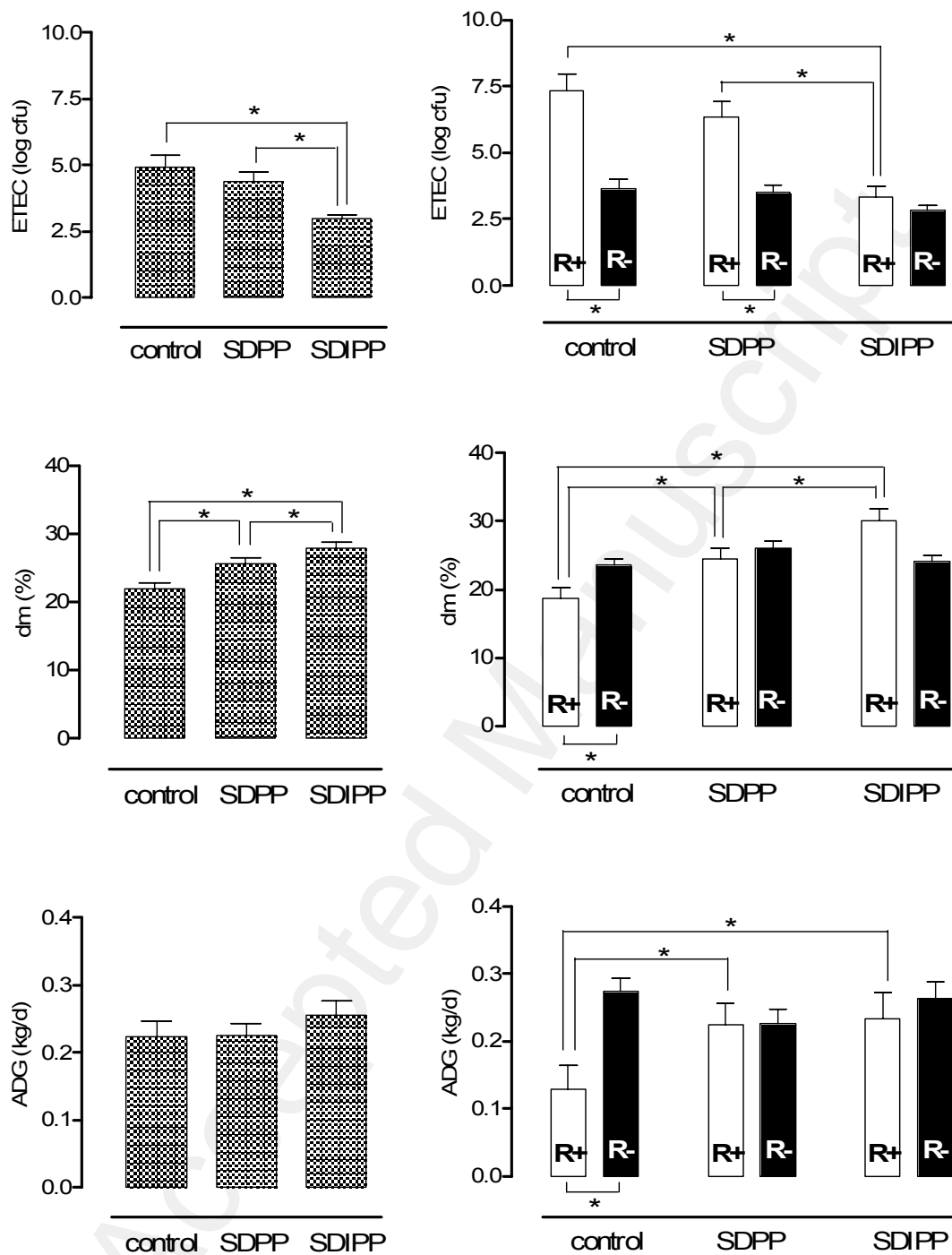


Table 1. Chemical analysis of diet, % on as fed basis

	Control	SDPP	SDPIP
Fat	5.7	4.6	4.7
Crude fibre	3.6	3.3	3.7
Moisture	10.9	9.7	9.3
Ash	6.3	4.9	4.9
Starch	34.8	40.4	41.7
Protein	17.8	17.6	17.9
IgG	n.d.	1.7	1.8

n.d.: not detectable

Table 2: Production of porcine plasma powder, and analysis of SDPP and SDPIP

A. Yield		
	SDPP	SDPIP
Blood (L)	1029	924
Plasma (L)	587	504
Plasma/blood (%)	57	55
Powder (kg)	20.4	20.3
B. Composition		
	SDPP	SDPIP
Total bacterial count (log)/g	2.5	<2.0
<i>Enterobacteriaceae</i> (log)/g	<1.0	<1.0
<i>Salmonella.spp</i> / 25g	0	0
Protein (%)	84.3	85.1
Dry matter (%)	96.7	97.2
Solubility (%)	93.9	96.5
Antimicrobial residue	negative	negative
Immunoglobulin (g/kg)	231	253
C: Anti-F4 immunoreactivity in SDPIP (n=2) in the production process		
	Reciprocal titer \pm s.d.	% Activity
Plasma (50%)	42,130 \pm 3,649	100
Plasma powder (100%)	59,780 \pm 2,086	71
Pellet (8%)	4,908 \pm 86	73