Antibody responses to a *Cryptosporidium parvum* rCP15/60 vaccine

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***Cryptosporidium parvum* is a zoonotic apicomplexa-protozoan pathogen that causes gastroenteritis and diarrhoea in mammals worldwide. The organism is transmitted by ingestion of oocysts, which are shed in faeces, and completes its lifecycle in a single host.[1] *C. parvum* is ubiquitous on dairy operations worldwide and is one of the leading causes of diarrhoea in calves on these farms.[2,3] Here, for the first time, we describe the antibody response in a large group of cows to a recombinant *C. parvum* oocyst surface protein (rCP15/60) vaccine and the antibody response in calves fed rCP15/60-immune colostrum produced by these vaccinated cows. Results of recent genotype surveys indicate that calves are the only major reservoir for *C. parvum* infections in humans.[4] Human *C. parvum* infections are particularly prevalent and often fatal in neonates in developing countries and to immunocompromised people, such as AIDs patients.[4] Drug therapy against cryptosporidiosis is limited and not wholly efficacious in either humans or calves[5], making development of an effective vaccine of paramount**

importance. To date, there is no commercially available effective vaccine against *C. parvum*, although passive immunization utilizing different zoite surface (glyco)proteins has showed promise.[6-9] All cows we vaccinated produced an antibody response to the rCP15/60 vaccine and the magnitude of response correlated strongly with the subsequent level of antibody in their colostrum. All calves fed rCP15/60-immune colostrum showed a dose-dependent absorption of antibody. Our results demonstrate that vaccination of cows with rCP15/60 successfully induces antibodies against CP15/60 in their serum and colostrum and that these antibodies are then well absorbed when fed to neonatal calves. With further research, this *C. parvum* vaccine may well be a practical method of conferring passive protection to calves against cryptosporidiosis. Furthermore, a specifically targeted immune-colostrum may be valuable in protection and treatment of immunocompromised human patients with cryptosporidiosis.

The genus *Cryptosporidium* is comprised of to date, 20 species, with various host-adaptations and has been found infecting 155 species of mammals. Arguably the most important of these *Cryptosporidium* spp. are *C. hominis*, which is host-adapted to humans, and *C. parvum* which infects many mammals and is an important zoonosis.[1,4,10-12] The life cycle of *Cryptosporidium* spp. results in production of an encysted stage (oocyst), which is passed in the faeces of the host. The oocyst is then transmitted by the faecal-oral route via contaminated water, food, fomites by direct contact with human or animal faeces. Cryptosporidiosis is the clinical syndrome of fever, diarrhoea, vomiting, abdominal pain, large volumes of fluid loss.[1] In humans, the species and subtypes of *Cryptosporidium*, source of infection and type of transmission

vary geographically.[10] Cryptosporidiosis (mainly due to *C. hominis*) is an important cause of morbidity and mortality of children in developing countries, through diarrhoea with subsequent dehydration and death.[13,14] In the developed nations, *C. parvum* most often infects immunosuppressed people such as AIDs patients but cryptosporidiosis outbreaks in the general immune-competent population have occurred due to contamination of a water supply.[10,14-16] The largest of these outbreaks was in Milwaukie, Wisconsin in 1993 with approximately 403,000 people affected.[17] Data collected via the National Dairy Heifer Evaluation Project indicated that *Cryptosporidium* spp. were detected in over 90% of North American dairy operations.[18] *C. parvum* is one of the leading causes of diarrhoea in neonatal dairy calves and as such, contributes to substantial economic losses.[2,3]

We are developing a *C. parvum* vaccine for use in pregnant cows to provide passive protection to the calf, via colostrum as an aid to prevention of diarrhoea caused by *C. parvum.* Development of vaccines centres round interruption of the lifecycle of *C. parvum* via antibodies that target critical surface exposed proteins and hence interrupting replication and survival of the organism. Over the past 20 years, some research groups have published data on passive immunization and immunotherapy against *C. parvum* in mice, goats and cattle using different zoite surface (glyco)proteins expressed during, and involved in, invasion and infection of host epithelial cells.[6-9, 19] However, a successful vaccine has yet to emerge.

The 15 kDa 123 amino acid antigen of *C. parvum* designated CP15/60 (GenBank Accession No. L34568) was identified by Jenkins and Fayer.[20] CP15/60 is expressed by the infective sporozoite and merozoite stages on the oocyst surface and is also associated with internal structures.[19] The gene encoding the CP15/60 has been

cloned, and expressed as a recombinant protein (rCP15/60).[20] We vaccinated pregnant 19 – 26 month-old heifers with rCP15/60 in a water-in-oil adjuvant or an adjuvant placebo. All heifers had low (< 8) CP15/60 antibody titres prior to vaccination. rCP15/60 vaccinated heifers had a significantly higher serum and colostrum titres to CP15/60 compared to control heifers at all 3 measured time points after vaccination ($P < 0.0001$, n = 40) (Table 1). The serum CP15/60 antibody titre at its highest point (day 42 after vaccination) had a strong positive correlation with the CP15/60 antibody titre in the colostrum (r = 0.82, $P < 0.0001$, n = 20 (Fig. 1).

Forty female and male Holstein dairy calves, unrelated to the vaccinated or control heifers, were randomly assigned and fed either rCP15/60-immune colostrum or control colostrum produced by the aforementioned vaccinated heifers. CP15/60 antibody titres were low (< 8) in all calves prior to colostral administration. In order to assess endogenous humoral immune response in the face of natural infection, specifically to CP15/60 antibodies in the face of natural infection, we administered 10^4 viable *C. parvum* oocysts orally to control calves at 12 hours of age. We found that calves administered rCP15/60-immune colostrum had significantly higher serum CP15/60 antibody titres at 48 hours, 96 hours and 21 days post colostral ingestion compared to calves administered the control colostrum ($P < 0.0001$, n = 39) (Table 2). Of the time points tested, calf serum CP15/60 antibody titre peaked at 48 hours after colostral ingestion. In the immune-colostrum fed calves, serum CP15/60 antibody titre at all three time points measured post ingestion was strongly correlated with the CP15/60 antibody titre in the actual colostrum aliquot (at 48 hours r = 0.83, $P < 0.0001$, at 96 hours r = 0.83, $P < 0.0001$, at 21 days, r = 0.78, $P < 0.0001$, n = 39 at all time points) (Fig. 2 a-c). This showed that the calves absorbed the colostrum well in a dose

dependent fashion. We confirmed that there was no significant difference in sex distribution or weight in the two groups of calves at enrolment in the study (birth) and thus these were not potential confounding factors ($P = 0.6$, n = 39).

In addition to its importance as a zoonosis, it is the difficulty in control of *Cryptosporidium* spp. that emphasises the need for a successful vaccine. Eradication of of *Cryptosporidium* spp. from the environment is extremely difficult, as the infective oocysts are resistant to most disinfectants, can persist viable in the environment for many months. The infective oocysts are also very small (4 – 6 µm in diameter), evading many municipal water filtering strategies.[1,4,15] Historically, drugs used to treat cryptosporidiosis have only been partially effective.[5] Recently, the anti-protozoal agent nitazoxanide has been demonstrated to be efficacious in cryptosporidiosis in humans[21] and experimentally in calves[22] but only when a normal immune response is present in the individual treated.[23] There are no drugs effective against *Cryptosporidium* spp. licensed for use in the dairy industry in the USA although halofuginone (labelled for use in Europe and Canada) and paramomycin (labelled for use in Europe) have partial efficacy.[24,25]

This is the first report of a CP15/60 vaccine against *C. parvum* tested on the cohort for which it is intended (i.e. vaccinated pregnant cattle with the immune-colostrum fed to commercial dairy calves with a normal sex distribution). In 1999, Jenkins et al. administered rCP15/60-immune colostrum produced by vaccinated cows to mice and demonstrated a partial protection against intestinal *C. parvum* infection in these mice compared to controls. However, these cows were vaccinated in a non-conventional manner by injection of recombinant plasmid DNA encoding the CP15/60 antigen directly in to the mammary gland.[26] We have shown that when administered the

CP15/60 vaccine, pregnant heifers successfully produce high levels of CP15/60 antibody in their colostrum. The CP15/60 antibody is then reliably absorbed by calves from the colostrum. We did not see a significant increase in CP15/60 antibody titre in the control calves, despite infection with *C. parvum*. This confirmed that the CP15/60 antibody titre rise in the vaccinate calves was due to administration of rCP15/60-immune colostrum. The next stage in our work is to determine the minimum amount of antibody required to control disease due to *C. parvum*.

If our CP15/60-immune colostrum is successful at preventing disease due to *C. parvum*, not only will it significantly reduce calf morbidity and mortality in the dairy industry but it may impact human disease control also. Since many cases of human cryptosporidiosis are zoonotic, most often from a bovine source,[4,11,12] reduction in shedding of *C. parvum* by cattle may reduce the incidence of human cryptosporidiosis due to *C. parvum*. Bovine colostrum has already been used as therapy for cryptosporidiosis in immunosuppressed patients and children with diarrhoea.[27,28] Specific immune bovine colostrum may provide more specific, targeted immunotherapy against *Cryptosporidium* spp.

Methods

Heifer vaccination. We vaccinated 46, 19 – 26 month-old pregnant heifers with rCP15/60 with a water-in-oil adjuvant or an adjuvant placebo. Six heifers either had inaccurate breeding dates or did not produce sufficient first milking colostrum to feed a calf and therefore were not used further in the study. Heifers were 180 to 210 days pregnant at the first vaccination, and were revaccinated 21 days later. The heifers calved 32 to 63 days (median, 71 days) after the second vaccination. Colostrum from the first milking (> 4 L) was collected on the day of parturition, aliquoted, labelled by

heifer identification number and stored at -10°C until use. We defrosted the colostrum aliquots as needed in a hot water (85-90 °C) bath, immediately prior to feeding to the calves.

Calves. Male and female Holstein calves weighing between 29.5 and 54.5 kg (65 - 120 lbs) were enrolled at birth from a commercial dairy herd. The calves were delivered, handled and housed such as to minimize exposure to environmental pathogens, including any environmental *C. parvum*. Immediately prior to parturition, we washed the dam's perineum and vulva with soap and warm water. We delivered each calf manually on to clean plastic sheeting so that no part of the calf made contact with the bedding or faeces and then brought the calf to an individual clean processing area. Each calf was identified with an ear tag number and a 5 mL blood sample was collected via jugular venipuncture for measurement of initial rCP15/60 titre. Calves were randomized to rCP15/60-immune colostrum or control colostrum, and fed 2 litres of the appropriate colostrum via oro-gastric tube feeder within 1 hour of birth. Calves were loose housed in individual box stalls bedded with wood shavings in a Biosecurity Level 2 facility at Cornell University. We fed a second 2 litres of either rCP15/60 immune colostrum or control colostrum 12 hours after birth via oro-gastric tube feeder. One hour after this second feeding, we administered 10^4 oocysts of *C. parvum* in 4 mLs of distilled water via an oesophageal tube feeder to the control calf group. Thereafter, we fed all calves 2 litres of commercial milk-replacer twice daily from a bucket with free choice water available at all times. Calves were bled for serum prior to the first colostrum feeding and 48 hours, 96 hours, and 21 days after the feeding. We separated the serum and stored it at -80 °C prior to ELISA analysis.

***Cryptosporidium parvum* oocyst purification.** To obtain *C. parvum* oocysts for infection of the calves, we collected faeces from 7 – 14 day old *C. parvum* infected Holstein calves from a large commercial dairy farm in New York State, USA. We processed the faeces to obtain *C. parvum* oocysts by continuous flow differential

density flow as previously described.[29] After a final Percoll purification step we washed the oocysts by centrifugation 3 times in cold distilled water at 2,100 g for 10 minutes to remove the Percoll, adjusted to a concentration of 1000 oocysts per mL with distilled water and stored at 4°C. Prior to inoculation into the calves, we assessed oocyst viability using a dye permeability assay.[29]

ELISA. We used a competitive ELISA to determine serum and colostrum antibody titres to CP-15/60. ELISA plates were coated with purified rCP15/60 antigen, blocked and washed. Serial two-fold dilutions of test bovine sera and dilutions of positive and negative control sera were made on low binding dilution plates. The diluted serum samples were transferred to the coated plate and co-incubated with rabbit anti-CP15/60 (the competitive serum). The plates were washed prior to incubation with horse radish peroxidise conjugated anti-rabbit serum. TMB (3,3',5,5'-Tetramethylbenzidine) dye-substrate was added to the wells and the plates then incubated. The colour reaction was stopped by the addition of acid and the optical densities read at 450 nm with a reference wavelength of 540 nm. The antibody titre was determined based on the reciprocal of the highest dilution showing approximately 30% inhibition of the competitive antibody. The values on each plate were adjusted based on the response of the positive control serum.

Statistics. Using the Shapiro-Wilk test, we determined the data to be non-Gaussian. We used the Wilcoxon Rank Sum test to compare sets of continuous data (weight and antibody titres) for the two groups of calves (vaccinates and controls) and Fisher's Exact test to analyse the dichotomous variable of sex. Associations between colostrum and serum antibody titres in both the cows and calves were analysed using Spearman's Rank Correlation. When looking at the antibody titres over the 3 different time points,

due to multiple comparisons within the same animal, a Bonferroni correction was used and hence alpha set at 0.017. For all other tests where multiple comparisons were not being made, alpha was set at 0.05. Data were anlyzed using Statistix 9.0 (Analytical Software, Tallahassee FL).

This study was approved by the Institutional Animal Care and Use Committee (IACUC) at Cornell University.

References

1. Fayer, R. Cryptosporidium: a water-borne zoonotic parasite. *Vet. Parasitol.* **126,** 37–56 (2004).

2. Trotz-Williams, L.A., Wayne Martin, S. Leslie, K.E., Duffield, T., Nydam, D.V. & Peregrine, A. S. Calf-level risk factors for neonatal diarrhea and shedding of *Cryptosporidium parvum* in Ontario dairy calves. *Prev. Vet. Med.* **82,** 12–28 (2007).

3. LeFay, D., Naciri, M., Poirier, P. & Chermette, R. Prevalence of Cryptosporidium infection in calves in France. Vet. Parasitol. **89,** 1–9 (2000).

4. Xiao, L. & Feng, Y. Zoonotic Cryptosporidiosis. FEMS *Immunol. Med. Microbiol.* **52,** 309–323 (2008).

5. Gargala, G. Drug treatment and novel drug targets against Cryptosporidium. *Parasite.* **15,** 275–281 (2008).

6. Fayer, R., Andrews, C., Ungar, B.L. & Blagburn, B. Efficacy of hyperimmune bovine colostrum for prophylaxis of cryptosporidiosis in neonatal calves. *J Parasitol.* **75,** 393–397 (1989).

7. Harp, J.A. & Goff J.P. Protection of calves with a vaccine against *Cryptosporidium parvum J. Parasitol.* **81,** 54–57 (1995).

8. Perryman, L.E., Kapil, S.J., Jones, M.L. & Hunt, E.L. Protection of calves against cryptosporidiosis with immune bovine colostrum induced by a *Cryptosporidium parvum* recombinant protein. *Vaccine*, **17,** 142–149 (1999).

9. Sagodira, A., Buzoni-Gatel, D., Iochmann, S., Naciri, M. & Bout, D. Protection of kids against *Cryptosporidium parvum* infection after immunization of dams with CP15-DNA. *Vaccine*, **17,** 2346–2355 (1999).

10. Xiao, L. Molecular epidemiology of cryprtosporidiosis: An update. *Exp. Parasitol.* [Eupub ahead of print] April 7 2009.

11. Ng, J., Eastwood, K., Durrheim, D., Massey, P., Walker, B., Armson, A. & Ryan, U. Evidence supporting zoonotic transmission of Cryptosporidium in rural New South Wales. *Exp. Parasitol.* **119,** 192–195 (2008).

12. Feltus, D.C., Giddings, C.W., Schneck, B.L., Monson, T., Warshauer, D. & McEvoy, J. M. Evidence supporting zoonotic transmission of *Cryptosporidium* spp. In Wisconsin. *J. Clin. Microbiol.* **44,** 4303–4308 (2006).

13. Mor, S.M. & Tzipori, S. Cryptosporidiosis in children in Sub-Saharan Africa: a lingering challenge. *Clin. Infect. Dis.* **47,** 915–921 (2008).

14. Tumwine, J.K., Kekitiinwa, A., Nabukeera, N., Akiyoshi, D.E., Rich, S.M., Widmer, G, Feng, X. & Tzipori, S. *Cryptosporidium parvum* in children with diarrhea in Mulago Hospital, Kampala, Uganda. *Am. J. Trop. Med. Hyg.* **68,** 710–715 (2003).

15. Tzipori, S. & Widmer, G. A hundred-year retrospective on cryptosporidiosis. *Trends Parasitol.* **24,** 184-189 (2008).

16. Huang, D.B., Chappell, C. & Okhuysen, P.C. Cryptosporidiosis in children. *Semin. Pediatr. Infect. Dis.* **15,** 253–259 (2004).

17. MacKenzie, W.R., Hoxie. N.J., Proctor, M. E., Gradus, M.S., Blair, K., A., Peterson, D.E., Kazmerczak, J.J., Addiss, D.G., Fox, K., R., Rose, J. B. & Davis, J. P. A Massive outbreak in Milwaukee of of Cryptosporidium infection transmitted through the public water supply. *N. Eng. J. Med.* **331,** 161–167.

18. USDA. *Cryptosporidium* is common in dairy calves. National Dairy Heifer Evaluation Project. Available at: http://nahms.aphis.usda.gov/dairy/#ndhep. Accessed Aug. 15, 2009.

19. Boulter-Bitzer, J. I., Lee, H. & Trevors, J.T. Molecular targets for detection and immunotherapy in *Cryptosproidium parvum*. *Biotechnology Advances,* **25,** 13–44 (2007).

20. Jenkins, M.C. & Fayer, R. Cloning and expression of cDNA encoding an antigenic *Cryptosporidium parvum* protein. *Mol. Biochem. Parasitol.* **71,** 149–152 (1995).

21. Rossignol, J.F., Ayoub, A. & Ayers, M.S. Treatment of diarrhoea caused by *Cryptosporidium parvum*: a prospective randomized, double-blind, placebo-controlled study of nitazoxanide. *J. Infect. Dis.* **184,** 103–106 (2001).

22. Ollivett, T.L., Nydam, D.V., Bowman, D.D., Zambriski J.A., Bellosa, M.L., Linden, T.C. & Divers, T.J. Effect of nitazoxanide on cryptosporidiosis in experimentally infected neonatal dairy calves. *J. Dairy Sci.* **92,** 1643–1648.

23. Abubabar, I., Aliyu, S.H., Arumugam, C., Usman, N.K. & Hunter, P.R. Treatment of cryptosporidiosis in immunocompromised individuals: systematic review and meta-analysis. *Br. J. Clin. Pharmacol.* **63,** 387–393 (2007).

24. Fayer R. & Ellis, W. Paramomycin is effective as prophylaxis for cryptosporidiosis in dairy calves. *J Parasitol.* **79,** 771–774 (1993).

25. Naciri, M., Mancassola, R., Yvoré, P. & Peeters, J.E. The effect of halofuginone lactate on experimental *Cryptosporidium parvum* infections in calves. *Vet. Parasitol.* **45,** 119–207 (1993).

26. Jenkins MC, O'Brien C, Trout J, Guidry A & Fayer R. Hyperimmune bovine colostrum specific for recombinant Cryptosporidium parvum antigen confers partial protection against cryptosporidiosis in immunosuppressed adult mice. *Vaccine,* **17,** 2453-2460 (1999).

27. Greenberg, P.D. & Cello, J.P. Treatment of severe diarrhea caused by *Cryptosporidium parvum* with oral bovine immunoglobulin concentrate in patients with AIDS. *J. Acquir. Immune Defic. Syndr. Human Retrovirol.*, **13,** 348–354 (1996).

28. Nord, J., Ma, P., DiJohn, D., Tzipori, S. & Tacket, C.O. Treatment with bovine hyperimmune colostrum of cryptosporidial diarrhea in AIDS patients. *AIDS,* **4,** 28–32 (1990).

29. Jenkins, M.B., Anguish, L.J., Bowman, D.D., Walker, M.J. & Ghiorse, W.C. Assessment of a dye permeability assay for determination of inactivation rates of *Cryptosporidium parvum* oocysts. *Appl. Environ. Microbiol.* **63,** 3844–3850 (1997).

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supervision of technical personnel and interpretation of results and comments on manuscript drafts. A.J.B. conducted and supervised the live animal field work, laboratory procedures and data interpretation at Cornell University and prepared the manuscript. T.C.L. and J.Z. conducted and supervised the live animal field work and laboratory work at Cornell University. G.C, A.B. and R.D are immunologists who prepared the antigen and immune colostrum at Intervet Schering-Plough Animal Health. D.D.B. provided advice on experimental design and laboratory work and comments on the manuscript.

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Figure 1 CP15/60 antibody (Ab) titres in the serum (x axis) at day 42 post vaccination showed a strong correlation (Spearman's Rank Correlation) with CP15/60 Ab titres in the colostrum at parturition (y axis) in heifers inoculated with the rCP15/60 *C. parvum* vaccine (r = 0.82, *P*< 0.0001).

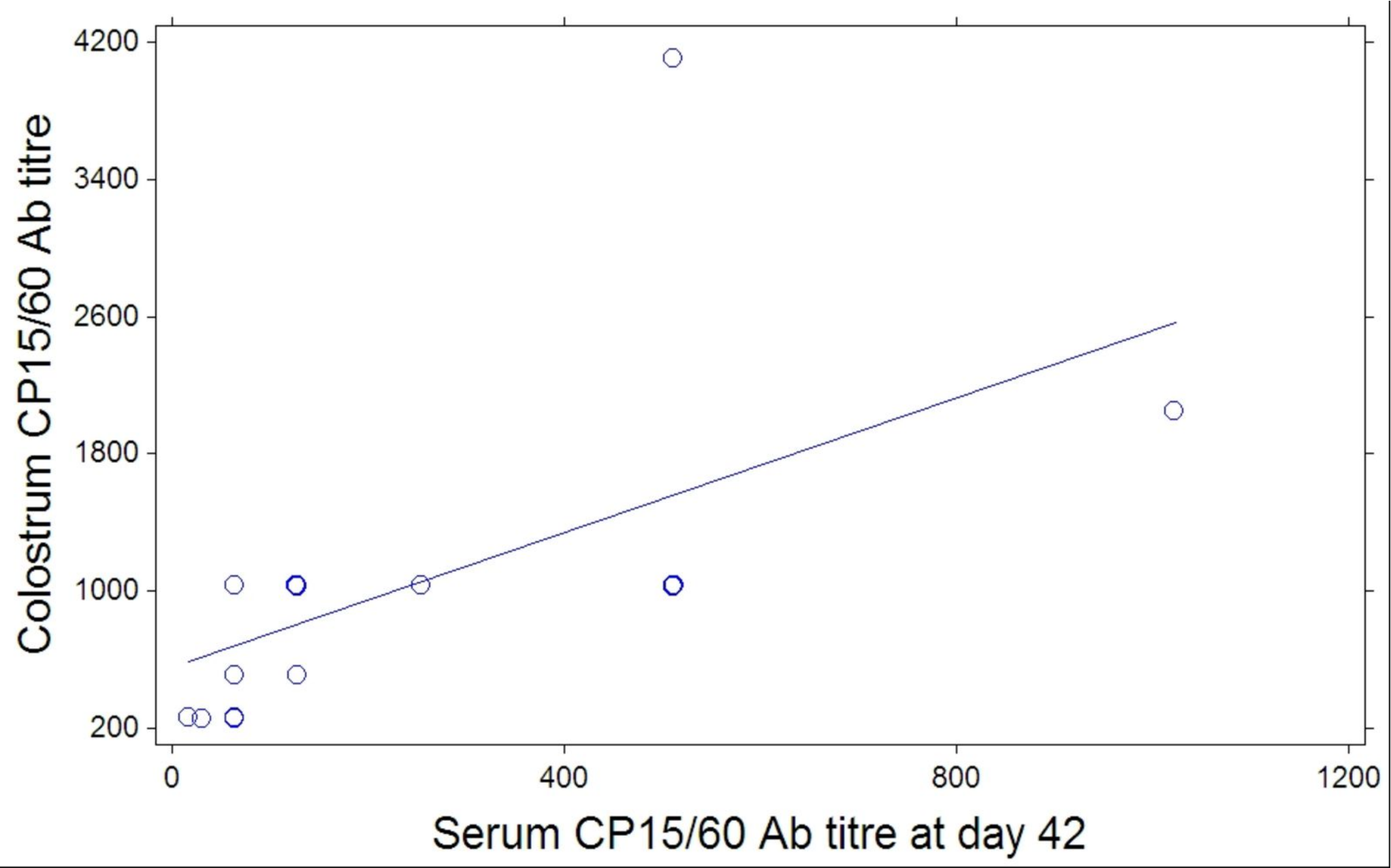
Figure 2 CP15/60 antibody (Ab) titre in the colostrum ingested (x axis) strongly correlated (Spearman's Rank Correlation) with CP15/60 antibody (Ab) titre in the serum of the calves that received CP15/60 immune-colostrum at all three time points measured. **a**, 48 hours post colostral administration (r = 0.83, p < 0.0001). **b**, 96 hours post colostral administration (r = 0.83, p < 0.0001). **c**, 21 days post colostral administration (r = 0.78, p = 0.0001).

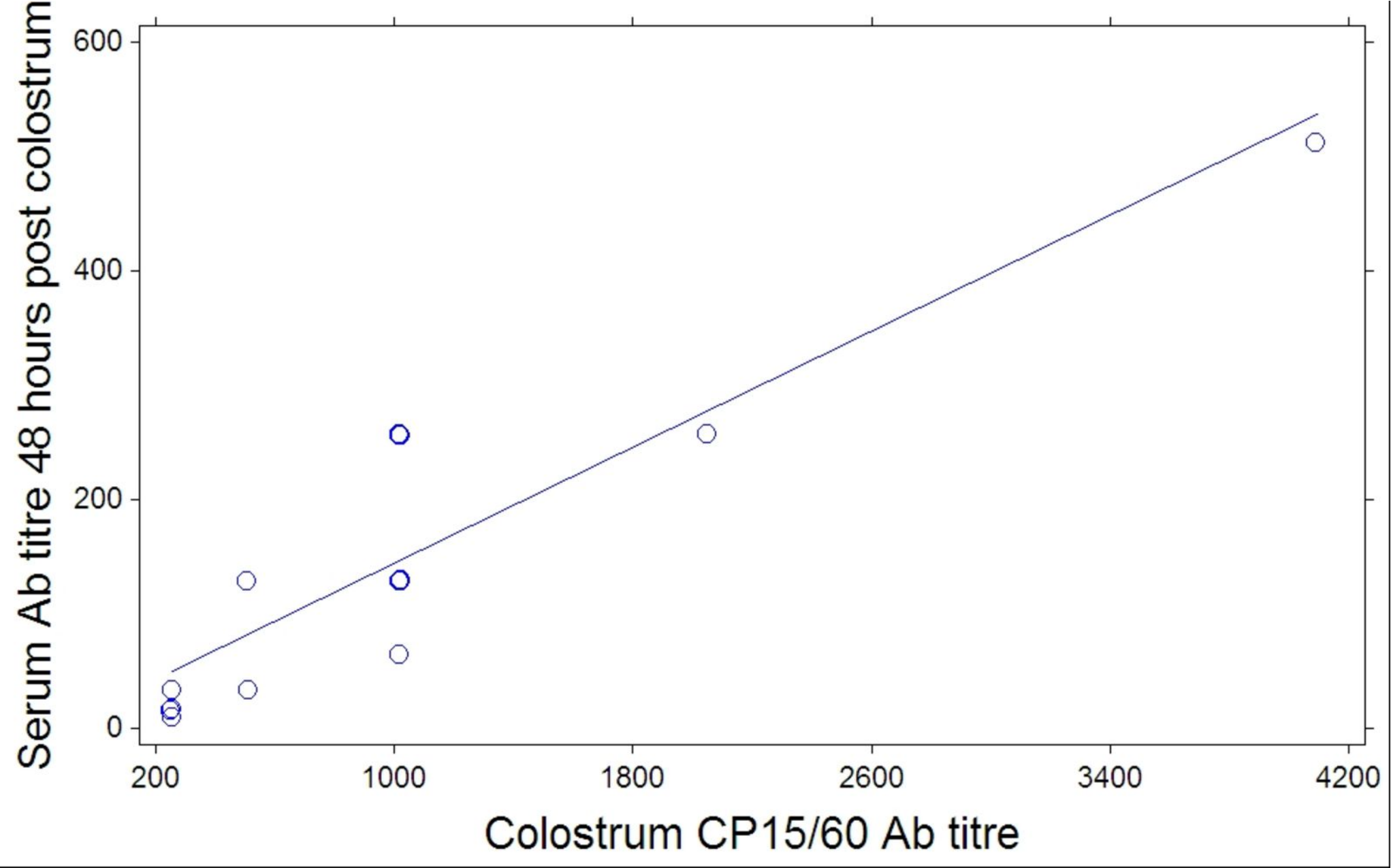
Table 1 CP15/60 antibody (Ab) titres post vaccination for all heifers

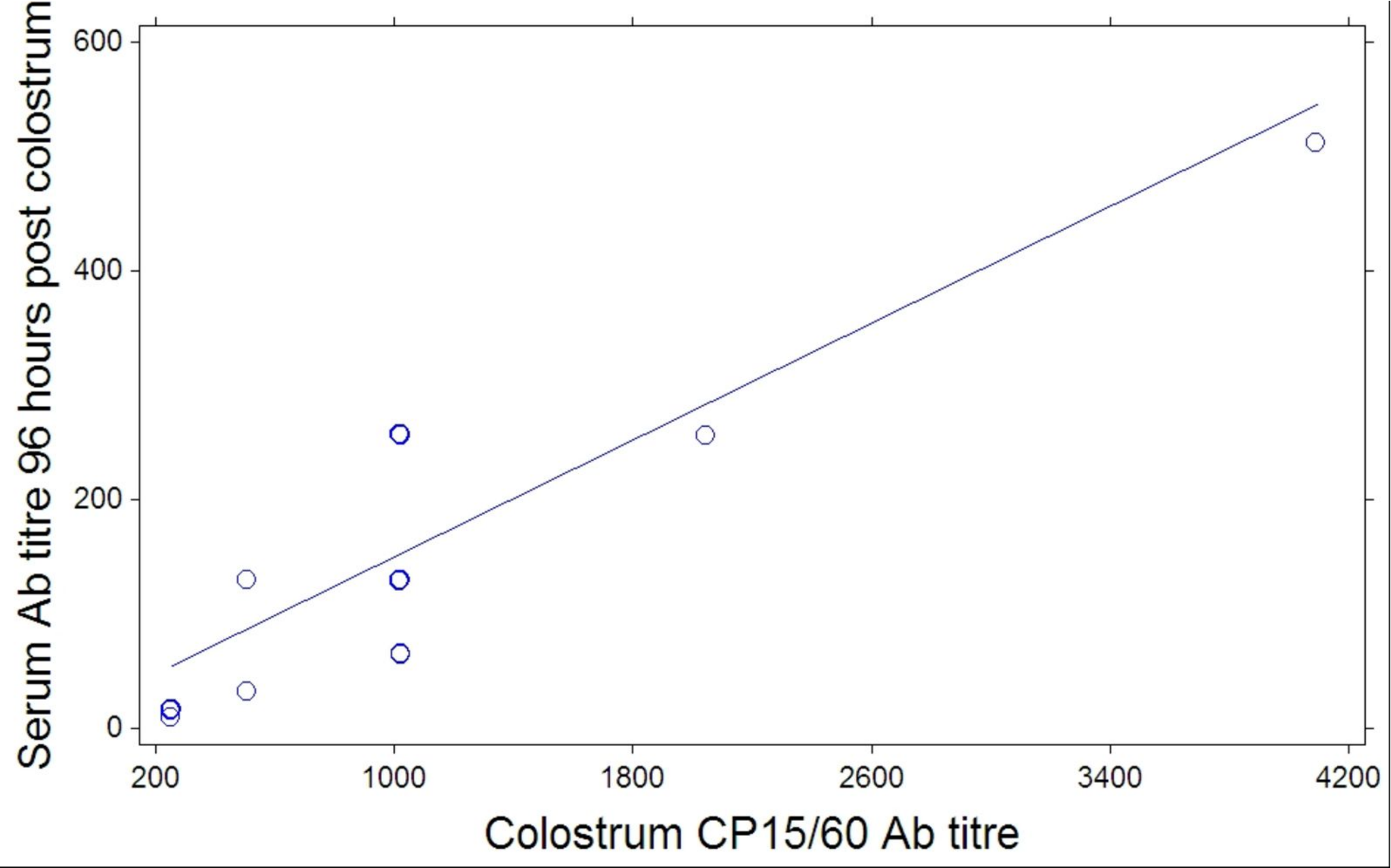
	Median (range) CP15/60 serum Ab titre at day 1	Median (range) CP15/60 serum Ab titre at day 42	Median (range) CP15/60 serum Ab titre, parturition	Median (range) CP15/60 Ab titre, 1st colostrum	Median (range) CP15/60 Ab titre, 2nd colostrum
Heifers given rCP15/60 (n = 20)	2 (< 2 – 8)	128 (16 – 1024)	32 (4 – 256)	1024 (256 –4096)	512 (64 – 2048)
Heifers given placebo (n = 20)	2 (< 2 – 4)	2 (< 2 – 64)	2 (< 2 – 4)	4 (<2 – 16)	2 (<2 – 16)

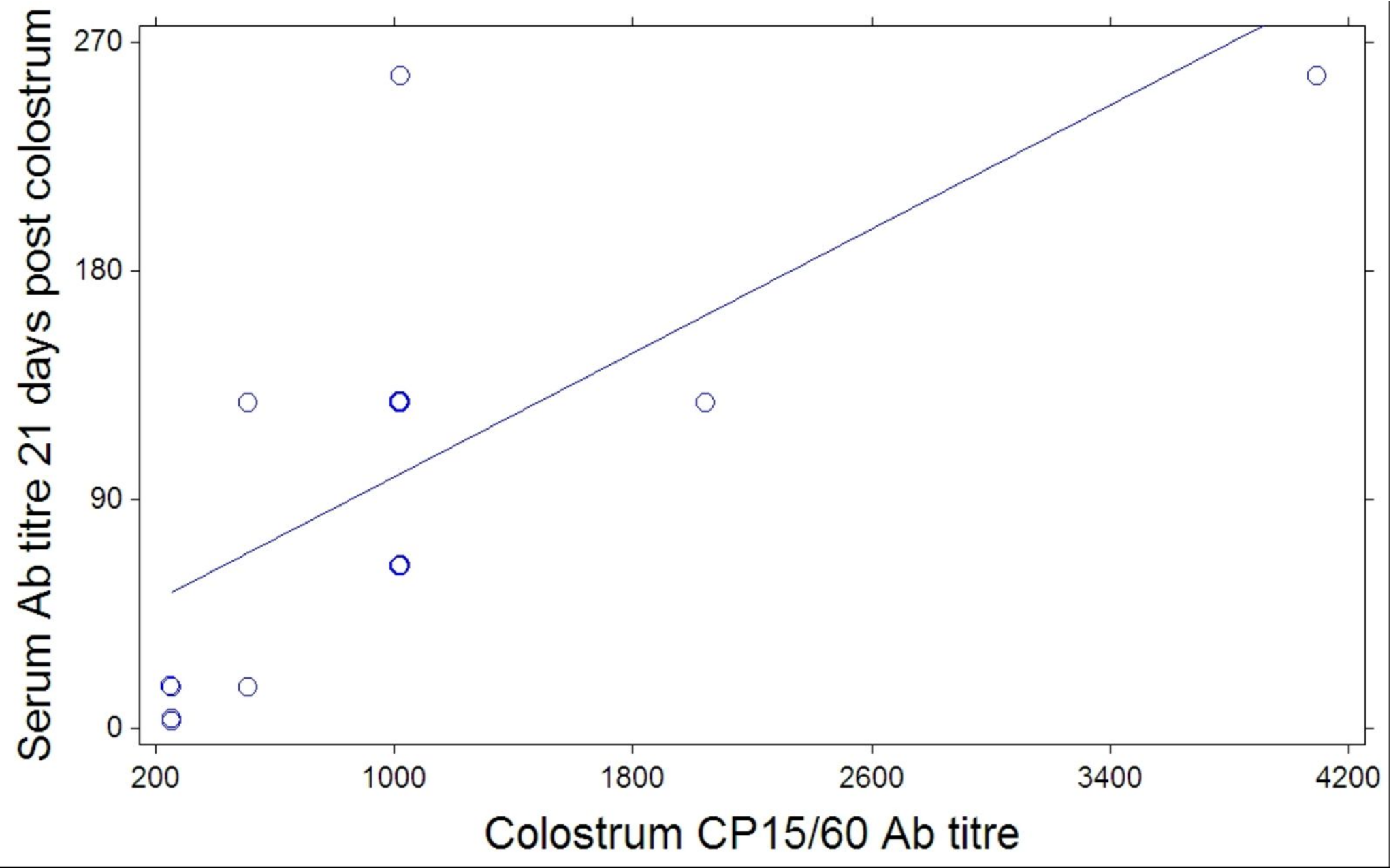
Table 2 Serum CP15/60 antibody (Ab) titres for all calves

	Median (range) CP15/60 Ab titre at birth (0 hours)	Median (range) CP15/60 Ab titre at 48 hours	Median (range) CP15/60 Ab titre at 96 hours	Median (range) CP15/60 Ab titre at 504 hours
Calves fed CP15/60-immune colostrum (n = 19)	< 2 (< 2 – 8)	128 (8 – 512)	128 (8 – 512)	128 (2 – 256)
Calves fed control colostrum (n = 20)	< 2 (< 2 – < 2)	< 2 (< 2 – < 2)	< 2 (< 2 – 2)	< 2 (< 2 – 128)


Colostrum CP15/60 Ab titre
4200
3400
2600
1800
1000
200
0
400
800
1200
Serum CP15/60 Ab titre at day 42


Serum Ab titre 48 hours post colostrum
600
400
200
0
200
1000
1800
2600
3400
4200
Colostrum CP15/60 Ab titre


Serum Ab titre 96 hours post colostrum
600
400
200
0
200
1000
1800
2600
3400
4200
Colostrum CP15/60 Ab titre


Serum Ab titre 21 days post colostrum
270
180
90
0
200
1000
1800
2600
3400
4200
Colostrum CP15/60 Ab titre