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SERUM LEVELS OF THE IMMUNOGLOBULINS IgG AND IgG(T) IN HORSES

By

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EK, NILS: *Serum levels of the immunoglobulins IgG and IgG(T) in horses.* Acta vet. scand. 1974, *15*, 609—619. — Levels of the immunoglobulins IgG and IgG(T) in serum in Norwegian horses of the breeds "Døle" and "Fjord" were determined by the quantitative radial immunodiffusion test.

No significant differences were apparent between the 2 Norwegian breeds. The immunoglobulin levels were approximately in the same range as previously reported for Shetland ponies.

Immunoglobulins could not be detected in the newborn foal. As early as 24 hrs. after birth the mean immunoglobulin level was within the adult range. After a drop during the first month of life, the immunoglobulins increased. IgG(T) rose more rapidly and to a higher level than IgG.

In 2 year old horses, IgG(T) was significantly higher than in adults, while IgG was significantly lower. IgG(T) seems to be a very important immunoglobulin in foals and young horses.

immunoglobulins; horse; breed; age differences.

The horse has been shown to possess at least 8 antigenically distinct immunoglobulins (*Rockey et al.* 1964, *Rockey* 1967). Amongst these is IgG, which includes the 3 subclasses IgGa, IgGb, and IgGc. However, a close relationship exists between these and especially between IgGa and IgGb which renders separation difficult (*Mc Guire et al.* 1972). Heavy chain analysis indicates that equine IgG is homologous to human and bovine IgG (*Prahl* 1966, *Milstein & Feinstein* 1968).

Subsequent work with equine-toxin sera demonstrated a 7 S antibody antigenically distinct from IgG and with a faster electrophoretic mobility. This antibody was called T-protein or T-component (*Nakamura & Katsura* 1964, *Nakamura & Nakamura* 1967) and was thought to correspond to human IgA (*Klinman et al.* 1966).

Weir & Porter (1966) noted cross reaction between the T-component and IgG. In addition, the C-terminal amino acid sequences of the heavy chains of the T-component and IgG were partly homologous. On the basis of these data it was concluded that the equine T-component represented an IgG subclass and the designation IgG(T) was adopted.

It has further been shown that IgG(T) may comprise as much as half the total immunoglobulin content in horses (*Mc Guire et al.* 1970).

Significant differences in the IgG(T) levels between horses of various breeds have been reported. Thus *Rouse* (1971) found lower levels in Thoroughbreds than in Shetland ponies.

In the present report, results from investigations of IgG and IgG(T) levels in healthy horses of the Norwegian breeds "Døle" and "Fjord" are described. The IgG includes the subclasses IgGa and IgGb.

MATERIALS AND METHODS

Blood samples (10 ml) were taken from the jugular vein. The blood was allowed to clot for 9 hrs. at room temperature, after which the serum was harvested and stored at — 20°C until analyzed.

Sera were obtained from the following categories of horses: Twenty "Døle" 4—12 years old, 20 "Døle" 3 years old, 15 "Døle" 2 years old, 12 "Døle" foals and 20 "Fjord" horses 4—12 years. Serum samples from the foals were examined at 12—48 hrs. after birth, then at weekly or monthly intervals up to the age of 12 months. Samples from 3 foals were also collected prior to the ingestion of colostrum and at 6 hrs. after birth.

Immunoglobulin purification

IgG was precipitated from normal equine serum with 40 % saturated ammonium sulphate, and further purified by DEAE-cellulose column chromatography in 0.1 M Tris-HCl-buffer, pH 8.0 (*Mc Guire et al.* 1972). The solution was dialyzed against isotonic saline for 24 hrs. and concentrated to about 1 % IgG by applying Aquaside (Calbiochem) as a dehydrating agent around the dialyzer tubing for 4 hrs.

IgG(T) was also isolated from the serum of a horse which had been hyperimmunized with tetanus toxoid (obtained from the National Veterinary Institute, Oslo) by precipitation with

40 % saturated ammonium sulphate. The precipitate was redissolved and dialyzed against 0.1 M Tris-HCl-buffer, pH 8.0, added to a DEAE-cellulose column, and eluted with the same buffer. After the initial peak, containing IgG, a NaCl gradient in the starting buffer from 0 to 0.5 M was applied. Those fractions eluted by the gradient which were rich in IgG(T) were pooled, dialyzed with 0.05 M sodium barbitol buffer, pH 8.6, and subjected to electrophoresis in a Pevikon block (*Müller-Eberhard* 1960). The IgG(T) from the Pevikon block electrophoresis was further purified by gel filtration on Sephadex G-150 with 0.15 M phosphate buffer, pH 7.4. Fractions of 5 ml were collected and concentrated to about 0.5 %.

In order to test the purity of the isolated IgG and IgG(T), these fractions were examined by immunoelectrophoresis carried out on 8×8 cm glass plates using 1 % agarose (L'industrie Biologique Francaise S.A. Gennevilliers Seine) with sodium barbitol buffer at 0.025 ionic strength and pH 8.6. Commercial rabbit anti-

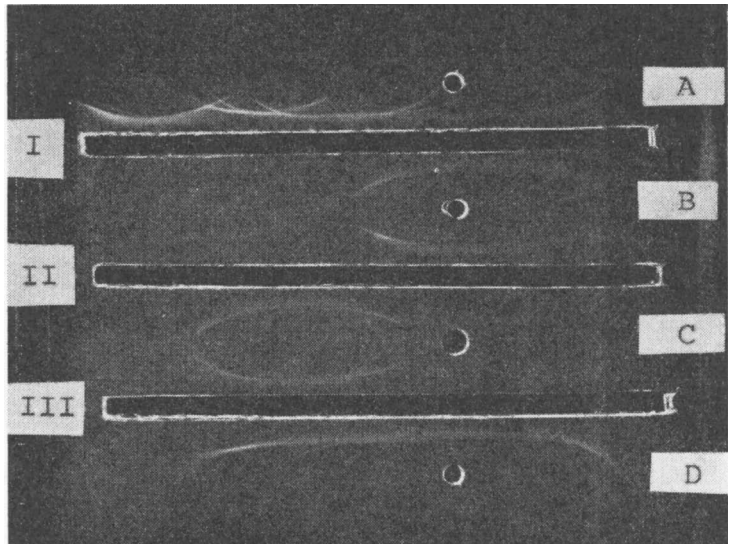

Figure 1. Immunoelectrophoretic characterization of purified equine immunoglobulins employed in the present investigation.

A and D: Normal horse serum.
B: IgG.
C: IgG(T).
Trough I and II: Anti-horse serum.
Trough III: Anti-γ horse serum.

horse serum and anti-γ horse serum (Behringwerke AG, Marburg-Lahn) were used. The antigens IgG and IgG(T) gave only 1 arc of precipitation on immunoelectrophoresis against antihorse serum (Fig. 1).

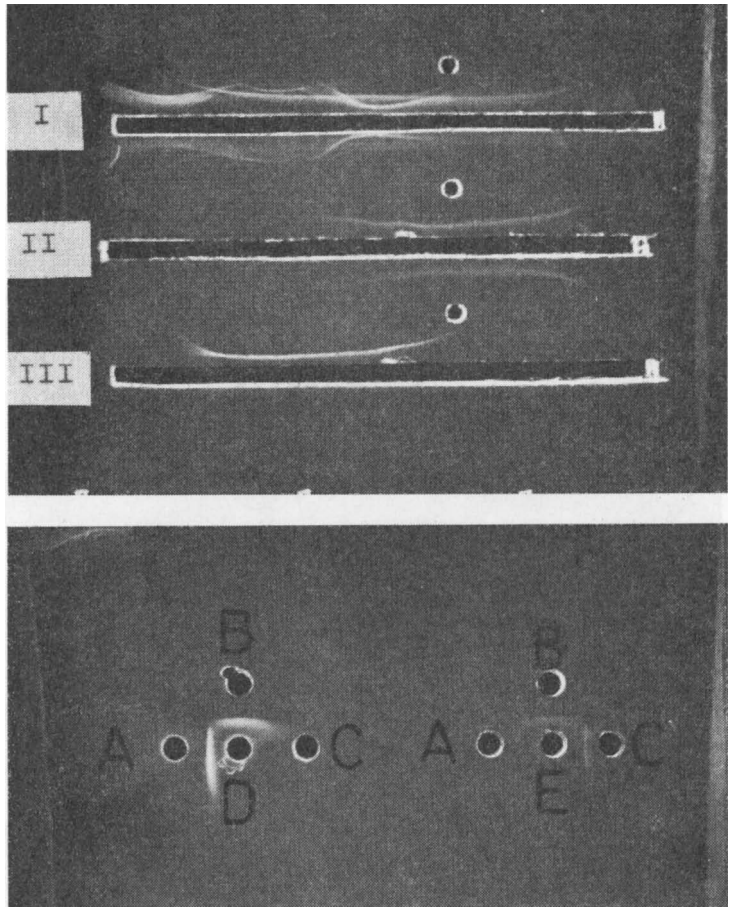

Figure 2. Immunoelectrophoresis and immunodiffusion tests of monospecific anti-IgG- and anti-IgG(T) serum.

Trough I: Anti-horse serum.
„ II: Monospecific anti-IgG serum.
„ III: „ anti-IgG(T) serum.
Wells: Normal horse serum.

Immunodiffusion:
A: IgG.
B: Normal horse serum.
C: IgG(T).
D: Monospecific anti-IgG serum.
E: „ anti-IgG(T) serum.

Preparation of antisera

Anti-horse IgG and IgG(T) sera were prepared by immunizing rabbits with 1 mg of pure IgG and IgG(T) (0.1 % solution) incorporated in an equal volume of Freund complete adjuvant (Difco Laboratories). Two subcutaneous injections were given at an interval of 10 days followed by 4 injections at weekly intervals. One week after the last injection 30 ml of blood was drawn from the ear vein.

To obtain monospecific antisera, the crude anti IgG serum was absorbed with the previously separated equine IgG(T) (0.5 % solution), in a proportion 2:1, and crude anti IgG(T) serum with equine IgG (1 % solution) in the proportion 3:2. The sera were shown to be monospecific on immunoelectrophoresis and immunodiffusion tests against anti-horse serum. The diffusion-in-gel technique by Ouchterlony was used (*Ouchterlony* 1968). Each antiserum showed only 1 single precipitation line (Fig. 2).

Quantity determination of immunoglobulins

The technique used was essentially as described by *Mancini et al.* (1965). Monospecific anti-IgG and anti-IgG(T) serum were preheated at 56°C and incorporated into agarose gel, 0.5 ml in 4.5 ml agarose. Five ml of the warm agarose-antiserum mixture was transferred to glass slides (7×7 cm). After solidification, wells, 3.0 mm in diameter, were punched out. They were filled with 5 µl of the serum dilution to be analyzed. The agarose was then placed horizontally in a humid chamber for 72 hrs.

The diameter of the precipitate was measured and the area of the precipitate was calculated according to the formula πr^2. The standard curve was drawn with the use of 3 dilutions of reference IgG (1 %), 1:1, 1:2, 1:4, and of reference IgG(T) (0.5 %), undiluted, 1:1, 1:2. Pure IgG (1 %) and IgG(T) (0.5 %) isolated as previously described were used as reference antigens. The nitrogen content of the reference antigens was determinated by the Micro-Kjeldal method, and nitrogen content × 6.25 was regarded as the protein concentration.

Duplicate determinations were made for every sample on different plates. The standard deviation of the double tests was calculated by the formula $s = \sqrt{\frac{\Sigma d^2}{2n}}$, where d is the difference between the double tests and n the number of double tests. The

precision of the method was found to be of the order of 10 %. For other statistical calculations, the usual methods were employed (*Snedecor & Cochran* 1967).

RESULTS

Serum immunoglobulin levels of IgG and IgG(T) in horses from the Norwegian breeds "Døle" and "Fjord" are given in Table 1.

In the adult horse the ratio between IgG and IgG(T) is about 3:2. In young horses, IgG is lower whereas IgG(T) is higher as compared with adult animals.

There were no significant differences between the 2 Norwegian breeds. IgG was significantly lower in 3 year old horses than in adults ($P < 0.05$), while there was no significant difference in IgG(T) between these 2 categories.

Two year old horses had a significantly lower IgG as compared with adults and 3 year olds ($P < 0.005$). The IgG(T) value in the 2 year old group was significantly higher than in the other categories ($P < 0.001$).

IgG and IgG(T) were not demonstrated in sera collected from foals prior to the ingestion of colostrum (Fig. 3).

Fig. 4 shows the mean serum concentration of IgG and IgG(T) in 3 "Døle" foals examined at various stages during the first 48

Table 1. Serum immunoglobulin levels of IgG and IgG(T) in Norwegian horses.

Breed and age	Number of animals	IgG concentration mg/100 ml			IgG(T) concentration mg/100 ml		
		mean	standard deviation	range	mean	standard deviation	range
A "Døle", adults	20	1470	158	1200—1770	948	209	570—1290
B "Fjord", „	20	1454	203	1050—1850	908	256	660—1560
C "Døle", 3 years	16	1330	221	780—1680	915	278	480—1560
D "Døle", 2 years	15	1070	196	690—1440	1286	432	570—2130

Comparison							
IgG				IgG(T)			
A—B	A—C	A—D	C—D	A—B	A—C	A—D	C—D
*n.s.	$P < 0.05$	$P < 0.005$	$P < 0.005$	*n.s.	*n.s.	$P < 0.01$	$P < 0.01$

*n.s. = not significant.

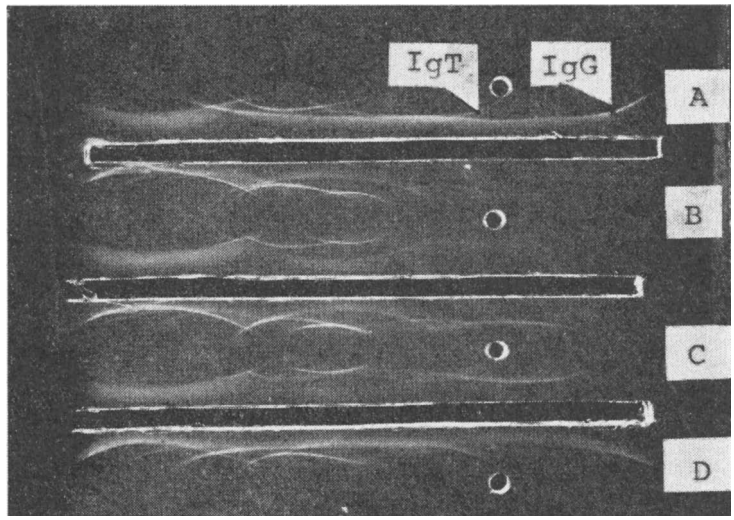

Figure 3. Immunoelectrophoresis of foal serum against anti-horse serum prior to and after the ingestion of colostrum.

A: Normal horse serum.
B: Foal serum at birth.
C: „ „ 6 hrs. after birth.
D: „ „ 12 „ „ „

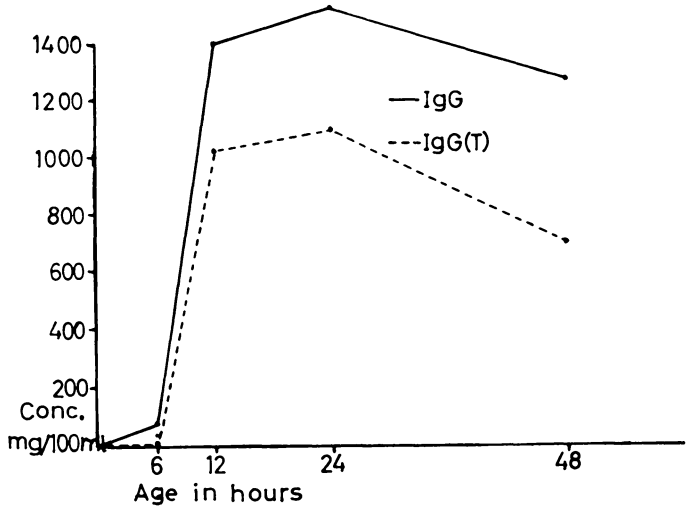

Figure 4. Mean serum concentration of IgG and IgG(T) in 3 "Døle" foals at various stages during the first 48 hrs. after birth.

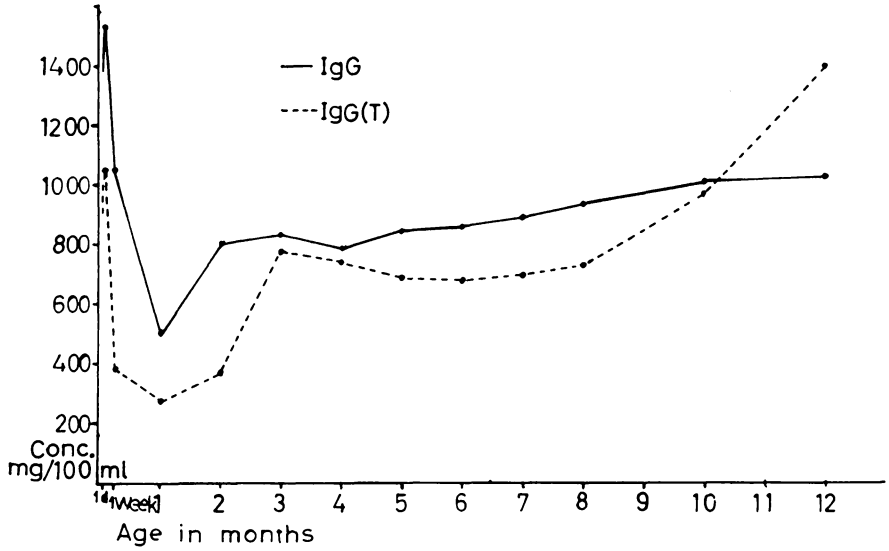

Figure 5. Mean serum concentration of IgG and IgG(T) in 12 "Døle" foals at intervals during the first year of life.

hrs. after birth. During the period 6—12 hrs. after birth, there was a rapid rise in both immunoglobulins, reaching a maximum at 24 hrs. At this time the mean levels of IgG and IgG(T) were about the same as in adult horses.

Fig. 5 illustrates IgG and IgG(T) in 12 "Døle" foals during the first year of life. After a peak at day 1, immunoglobulin levels fell markedly during the first month. During the period 1 to 3 months after birth levels of both IgG and IgG(T) again increased, and then remained more or less constant during the next few months. After 6 months IgG(T) levels began to rise noticeably, reaching a value at 12 months markedly above the adult mean level. As regards IgG, levels increased very slowly from 4 months of age onwards.

DISCUSSION

Rouse (1971) compared IgG and IgG(T) in Shetland ponies with Thoroughbreds. He found approximately the same IgG levels in the 2 groups. IgG(T) was however significantly higher in pony sera. It was postulated that this disparity could be the result of genetic breed differences.

Concerning the difference in IgG(T) between ponies and Thoroughbreds, it was stressed that the 2 groups were subjected to different feeding and management regimes. The ponies were at pasture and were not being worked, while the Thoroughbreds were racing and were being fed a high energy carbohydrate diet. Environmental or husbandry factors might therefore influence immunoglobulin levels.

No significant differences between the 2 Norwegian horse breeds as to IgG and IgG(T) levels were found. In this connection it can be mentioned that the 2 Norwegian breeds of horses live under the same climatic conditions and are subjected to similar feeding and management.

Mc Guire et al. (1972), testing 20 Shetland ponies, reported values in the same range as in the present work.

Several workers have demonstrated that the newborn foal lacks detectable immunoglobulins prior to the ingestion of colostrum (*Morgan* 1972, *Rouse*). It is obvious also from the present investigations that foals absorb massive quantities of immunoglobulins during suckling, since the level at 24 hrs. is within the adult range.

The decrease in immunoglobulin concentration during the first month of life reflects the fact that foals do not synthesize immunoglobulins at this time. The increase in IgG and IgG(T) levels after 1 month indicates that the young foal starts to synthesize these immunoglobulins at this time. There was a period from 3 months of age when the synthesis of the immunoglobulins was in slight negative balance in relation to catabolism. This phenomenon is difficult to explain. In the investigation of *Morgan* there was also a slight decrease in the IgG value from the age of 112 days to 140 days.

It is striking that from the age of about 8 months, IgG(T) increased much more rapidly than IgG. Results indicate that from this age onwards the IgG(T) system in foals is more active than the IgG system. *Rouse* reported similar findings when investigating Shetland pony foals. The IgG system in the young horse seems to mature very slowly, not reaching the adult level until the age of 3—4 years.

The high IgG(T) level in young horses is remarkable. The results indicate that the IgG(T) fraction is a very important immunoglobulin in young horses.

The IgG(T) fraction has a faster electrophoretic mobility

than IgG. Therefore when using traditional zone electrophoresis, this fraction migrates close to many other globulins. Consequently, the separation and determination of IgG(T) using this method will probably be inaccurate. Thus zone electrophoresis is not suitable for the purpose of investigating immunoglobulin levels in the horse, especially in foals and young horses.

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REFERENCES

Klinman, N. R., J. H. Rockey, G. Frauenberger & F. Karush: Equine anti-hapten antibody. III. The comparative properties of γ G- and γ A-antibodies. J. Immunol 1966, *96*, 587—595.

Mancini, G., A. O. Carbonara & J. F. Heremans: Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 1965, *2*, 235—254.

McGuire, T. C., L. E. Perryman & J. B. Henson: Immunoglobulin composition of the hypergammaglobulinemia of equine infectious anemia. Fed. Proc. 1970, *29*, 435.

Mc Guire, T. C., T. B. Crawford & J. Henson: The isolation characterization, and functional properties of equine immunoglobulin classes and subclasses. Proc. 3rd Conf. Equine Infectious Diseases, Paris 1972, pp. 364—381 (Karge, Basel 1973).

Milstein, C. P. & A. Feinstein: Comparative studies of two types of bovine immunoglobulins G heavy chains. Biochem. J. 1968, *107*, 559—564.

Morgan, D. O.: Serum proteins of neonatal foals. Changes in electrophoregrams and total protein of foals blood serum from birth through five months of age. Proc. 3rd Conf. Equine Infectious Diseases, Paris 1972, pp. 410—418 (Karge, Basel 1973).

Müller-Eberhard, H. J.: A new supporting medium for preparative electrophoresis. Scand. J. clin. Lab. Invest. 1960, *12*, 33—37.

Nakamura, H. & T. Katsura: Immunochemical studies on diphtheria antitoxin. VI. Comparative studies of horse T and γ antitoxins in the quantitative precipitin reaction complement fixation, and indirect hemagglutination of tanned and toxin-coated erythrocytes. Jap. J. exp. Med. 1964, *34*, 167—196.

Nakamura, J. & M. Nakamura: The reaction of diphtheria toxin with horse antitoxin in the region of antibody excess. Jap. J. exp. Med. 1967, *37*, 447—460.

Ouchterlony, O.: Handbook of Immunodiffusion and Immunoelectrophoresis. Ann Arbor Michigan. Ann Arbor Science Publ. 1968.

Prahl, J. W.: In discussion on chemistry and biology of immunoglobulins. Proc. roy. Soc. B. 1966, *166*, 220.

Rockey, J. W.: Equine anti-hapten antibody. The subunits and fragments of anti-β-lactoside antibody. J. exp. Med. 1967, *125*, 249—275.

Rockey, J. H., N. R. Klinman & F. Karush: Equine anti-hapten antibody. I. 7 S β 2 A and 10 S γ 1-globulin components of purified anti β-lactoside antibody. J. exp. Med. 1964, *120*, 589—609.

Rouse, B. T.: The immunoglobulins of adult equine and foal sera: A quantitative study. Brit. vet. J. 1971, *127*, 45—52.

Snedecor, O. W. & W. G. Cochran: Statistical Methods. The Iowa State College Press, Ames, Iowa 1967.

Weir, R. C. & R. R. Porter: Comparison of the structure of the immunoglobulins from horse serum. Biochem. J. 1966, *100*, 53—58.

SAMMENDRAG

Serumkonsentrasjoner av IgG og IgG(T) hos hest.

Det ble foretatt bestemmelse av serumkonsentrasjoner av immunglobulinene IgG og IgG(T) hos voksne norske hester av rasene „dølehest" og „fjording" ved kvantitativ radial immundiffusjonstest. Av „dølehest" ble det også gjort undersøkelse på hester 2 og 3 år gamle og på en gruppe føll på forskjellig alderstrinn etter fødsel.

Signifikant forskjell mellom de to norske hesteraser kunne ikke påvises. Immunglobulinkonsentrasjonene var omtrentlig på samme nivå som tidligere er angitt for Shetland ponnier.

Immunglobuliner kunne ikke påvises hos det nyfødte føll. Allerede 24 timer etter fødsel var middelkonsentrasjonene av immunglobuliner på høyde med det voksne dyrs. Etter et fall i løpet av første levemåned ble det en stigning i immunglobulinene. IgG(T) steg raskere og til et høyere nivå enn IgG.

Hos 2 år gamle hester var IgG(T) signifikant høyere enn hos voksne hester, mens IgG var signifikant lavere. IgG(T) synes å være et meget viktig immunglobulin hos føll og unghester.

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