Quantitative Studies on Immunoglobulins and Transferrin in Equine Serum

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Abstract. Normal adult horse serum was analyzed by electrophoretic and immuno-electrophoretic methods. Seven different protein bands were found by cellulose acetate electrophoresis, 20 fractions by polyacrylamide gel electrophoresis, and a total of 20 antigenic components by immunoelectrophoresis developed with antiserum to normal adult horse whole serum.

Changes in the serum proteins, especially the immunoglobulins, IgG, IgG(T), and IgM, and transferrin, with the advance in age were determined by electrophoretic analyses and a quantitative immunodiffusion method. Precolostral foal serum was almost entirely deficient of immunoglobulins. Very soon after the first ingestion of colostrum, the levels of IgG(T) and IgM of the foal reached a maximal level, which was lower than the mean adult level, while the IgG level of the foal generally exceeded the adult level. Transferrin level was within the adult range in the precolostral serum. It showed no significant changes with the advance in age. On the other hand, all the three immunoglobulin classes and transferrin were high in level in the colostrum collected immediately after parturition, but decreased in level rapidly with the lapse of time after parturition.

At least six classes of immunoglobulins, namely, IgGa, IgGb, IgGc, IgG(T), IgM and IgA, have been individualized in horse serum and exocrine secretions by numerous studies [19–21, 23, 24, 31–36, 37, 38, 40, 41]. However, there is very little information on the transmission of passive immunity in the horse or the change of serum proteins in newborn foals [5]. Rouse [34] reported that no immunoglobulins were detectable in foal serum prior to the ingestion of colostrum, but that soon after suckling the levels of IgG, IgG(T) and IgM rose to approach the adult ranges.

Electrophoretic analyses, especially by cellulose-acetate, disc, and immuno-electrophoresis, and immunodiffusion methods have generally been used as diagnostic means in the human medicine. In the field of equine medicine, they may be used for the diagnosis of various diseases, but knowledge is much less complete on the significance and identification of the changes of all the components of horse serum proteins in the normal or various pathological conditions. Hence, it is considered of interest to study the identification of electrophoretic patterns of adult horse serum protein, and also to follow the normal changes of serum and colostrum protein fractions and the levels of the immunoglobulins, IgG, IgG(T) and IgM, and

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transferrin during various stages after birth.

Materials and Methods

Serum: Blood samples were collected from 45 horses, which consisted of 7 Anglo-Arabian and 38 Thoroughbred horses, and which ranged from 7 months to 27 years of age. The animals were free from obvious signs of any clinical disease and apparently normal in the physical examination, when blood samples were collected. Blood samples were also collected from 2 newborn foals prior to the ingestion of colostrum, 1, 3, 6, 12, 24 and 36 hours, and 3.7 and 30 days after birth, and then at monthly intervals up to 6 months of age.

Blood samples were taken from the jugular vein in all the animals, except precolostral newborn foals in which blood was obtained from the umbilical cord, by means of disposable syringe and needle. Serum was separated from each sample by centrifugation at 3,000 rpm for 30 minutes, frozen, and kept at −20°C before analysis was done.

Colostrum and milk: Equine milk samples were collected from mares immediately after parturition and 36 hours, 3 days, one week, one month and two months after parturition. Colostral and milk samples were centrifuged at 10,000 rpm for 30 minutes. An appropriate clear fraction was collected from them, adjusted to pH 4.6 to precipitate residual casein, and then recentrifuged in the same manner as before to remove casein and clarify further. The resultant whey was stored at −20°C before use.

Purification of immunoglobulins and transferrin: IgG, IgM and transferrin were isolated from normal horse serum, and IgG(T) was from the serum of a horse hyperimmunized against anthrax by anion exchange chromatography on columns of DEAE cellulose and/or gel-filtration on Sephadex G-200. The following modified procedures, essentially the same as described in previous studies [31-33, 57] were used.

IgG: Before being subjected to DEAE cellulose chromatography, horse serum (100 ml) was preliminarily salted-out in the following manner. An equal volume of PBS was added to the horse serum. Two hundred ml of saturated ammonium sulfate solution, pH 7.0, was added to the diluted serum, which was centrifuged. The resulting precipitate was dissolved in 100 ml of PBS. Then 25 ml of saturated ammonium sulfate solution was added to the resulting solution to give a 20% saturation, in which fibrinogen was precipitated. To the supernatant was added about 18 or 19 ml of saturated ammonium sulfate solution to give a 30% saturation, and centrifuged. The precipitate was dissolved in 100 ml of PBS. The same treatment was repeated three times. The resultant precipitate was suspended in a small volume of PBS, and dialyzed first against PBS and then against 0.005 M phosphate buffer, pH 7.4.

The sample obtained by the salting-out method mentioned above was fractionated by DEAE cellulose chromatography with a 1.5×40 cm column. A fraction (5 ml) was eluted with 0.005 M phosphate buffer (pH 7.4) at a flow rate of 8 ml/hr. The protein profile was ascertained by monitoring the eluates at 280 nm. A single peak was obtained. The fraction corresponding to the peak was collected, dialyzed, concentrated, and subjected to starch block electrophoresis in order to remove a small amount of β-globulin contained in the peak. The extracted γ-globulin, or crude IgG, was subjected again to DEAE cellulose chromatography under the same conditions. The resulting eluates contained no detectable proteins other than IgG.

IgG(T): Horse serum (100 ml) was salted out in 40%, saturated ammonium sulfate solution by the same procedure described above. After centrifugation, the precipitate was dissolved in PBS and dialyzed against 0.005 M phosphate buffer, pH 7.4. Then fractionation was carried out by stepwise elution chromatography on DEAE cellulose using 0.005 M phosphate buffer, pH 7.4, and 0.05 M NaH₂PO₄. From the eluates obtained with the latter buffer, the β-globulin fraction was separated by starch block electrophoresis. It was subjected again to DEAE cellulose column chromatography by the stepwise elution method which used four buffers: 0.005 M (pH 7.4) and 0.02 M (pH 6.0) phosphate buffer, and 0.02 M and 0.05 M NaH₂PO₄. A crude preparation of IgG(T) was obtained in the last peak eluted from the column, which also contained a very small amount of IgM.

The crude preparation of IgG(T) was purified by gel filtration on a column of Sephadex G-200 with 0.1 M tris-HCl, pH 8.0, containing 0.5 M NaCl.

IgM: A crude β-globulin fraction was obtained from horse serum (100 ml) by precipitation with 45% saturated ammonium sulfate solution, pH 7.0. It was separated further into a pure β-globulin and a γ-globulin fraction by starch block electrophoresis. The extracted γ-globulin fraction was eluted by reverse-flow exclusion chromatography (at a flow rate of 10 ml/hr) on a 2.8×100 cm column of Sephadex G-200 after extensive dialysis against 0.5 M NaCl containing 0.1 M tris-HCl buffer, pH 8.0, which was used for elution.

The first half of the first peak was pooled, con-
centrated, gel-filbrated again on Sephadex G-200, and eluted with the same buffer. A single peak was obtained and the fractions corresponding to the first half of this peak gave pure IgM.

Transferrin: Horse serum (50 ml) was precipitated with 0.4% rivanol (150 ml, pH 9.0). The supernatant was then concentrated, dialyzed against 0.05 M tris-HCl buffer (pH 8.0), and eluted by gradient elution chromatography on DEAE Sephadex A50 with 0.05 to 0.5 M tris-HCl buffer at pH 8.0. The fractions corresponding to the third peak were pooled, concentrated, dialyzed, and gel-filbrated on Sephadex G-200 under the same conditions as described in the procedure of purification of IgM. The first half of the second peak contained pure transferrin.

Purity of these four antigens was confirmed by disc electrophoresis and by immunoelectrophoretic analysis (Fig. 2).

Antiserum preparations: Antiserum against horse whole serum was prepared in rabbits by intracutaneous inoculation with horse serum emulsified in an equal volume of Freund’s complete adjuvant. Four weeks after the initial inoculation, the serum was injected intravenously into these rabbits 4 times at one-week intervals. The rabbits were bred one week after the final inoculation.

Anti-IgG serum was prepared by using washed precipitates from IgG purified by immunoelectrophoresis against anti-whole serum, as described by Nariuchi [26]. Rabbits were immunized by injecting intracutaneously with the antigen emulsified in complete Freund’s adjuvant, and by injecting intravenously with only the antigen 4 weeks after the initial inoculation. Immunoelectrophoretic analysis of horse whole serum using this antiserum showed only one precipitin line of IgG.

Antiserum against IgG(T). IgM or transferrin was also prepared in rabbits with the respective purified antigen in the same manner as described above. Absorption of antiserum against IgG(T) or IgM with isolated IgG produced specific antiserum against a single component in serum.

All the antisera were preserved in thimerosal (1:10,000) at −20°C.

Analytical procedure: Immunoelectrophoresis using agarose was performed by the method proposed by Grabar and Williams and modified by the authors [27]. Cellulose acetate electrophoresis was carried out by the Oxo2d membrane following the established procedure. Polyacrylamide gel (disc) electrophoresis was conducted by the modified method of Davis [7]. Single radial immunodiffusion was done by the method of Fahey and McKeveley [11] to determine the levels of several immunoglobulins and transferrin. Total protein concentration was determined by the biuret method.

To identify precipitin lines or bands observed by several electrophoretic analyses, some samples were stained with Sudan black B, Schiff’s reagent, paraphenylenediamine, and o-dianisidine, in addition to the routine protein staining with ponceau 3R or amidoblack 10B.

Results

Serum protein fractionation

Normal adult horse serum was divided into 20 fractions by the disc electrophoretic method with polyacrylamide gel and into seven different protein bands by cellulose acetate electrophoresis performed by the conventional methods. When the eluates of each band revealed by cellulose acetate electrophoresis were analyzed further by disc electrophoresis, each band was found to be a mixture of many different proteins. Especially, it was observed that IgG migrated primarily into the γ-globulin band, IgG(T) into the β2-band, IgM into the region between the β2- and γ-band, and transferrin into the β1-band. It was also demonstrated that the β2γ-globulin band separated by cellulose acetate electrophoresis contained a large amount of IgG(T) and low concentrations of transferrin, IgG and macroglobulin.

Immunoelectrophoretic patterns developed with the authors’ best antiserum against normal adult horse whole serum indicated the presence of at least 20 antigenic components; that is, two lines of prealbumin, one line in the albumin fraction, three in the α1-globulin fraction, four in the α2-globulin fraction, three in the β1-globulin fraction, four in the β2γ-globulin fraction, and three in the γ-globulin fraction (Fig. 1). Especially, three precipitin lines observed in the γ-globulin fraction were assumed to correspond to IgGa, IgGb,
and IgGc, respectively, as described by Rockey [32]. The authors’ isolated IgG corresponded to IgGa because of the characteristic shape of its precipitin line. In the β2-globulin fraction, a predominant line migrating faster than IgGc was identified as IgG(T), and a faint, characteristic line migrating a little slower than IgG(T) as IgM by using the isolated antigens, IgG(T) and IgM, respectively (Fig. 3). One of the remaining two lines, or a faint line presented between IgG(T) and IgM, was assumed to correspond to IgA described by Vaerman et al. [38] because of a little slower immunoelectrophoretic mobility than that of IgG(T) and its uniform occurrence. The other line was not identified distinctly either, but its characteristic staining property with Sudan black B suggested that it might be constituted by β2-lipoprotein. Transferrin was presented as the most intense line of the three noticed in β1-globulin fraction (Fig. 3).

Fig. 1 is the diagrammatic presentation of all the antigenic constituents revealed by immunoelectrophoretic analysis of normal adult horse serum protein.

Quantitative analysis

Investigation was carried out by various electrophoretic methods and radial immunodiffusion analysis. In precolostral newborn foal serum, deficiency in immunoglobulin was quite evident from the disc electrophoretic pattern and the immunoelectrophorogram developed with anti-normal adult horse serum (Figs. 4 and 5). Quantitative assays by the immunodiffusion reaction failed to detect IgG, IgG(T) and IgM in a concentration higher than 3.5, 13 and 3 mg/100 ml, respectively.

Very soon after the first ingestion of colostrum, however, the presence of all the classes of immunoglobulins was quite evident from the immunoelectrophorograms of these immunoglobulins. The results of studies on the quantitative aspects of IgG, IgG(T), IgM, in relation to the age of life are presented in Fig. 2. It may be seen from this figure that IgG began to increase in level distinctly 3 hours after the first colostrum ingestion and reached a maximal level at about one day of age. Thereafter, the IgG level declined markedly for the ensuing 2 months and then gradually until about five months of age, when there was again a slight increase in this level.

It may also be seen from Fig. 2 that the IgG(T) or IgM profile is generally similar to, but not identical with, that of IgG. At about one day of age, IgG(T) and IgM
reached a maximum level, which was lower than the mean adult level. Then the IgG(T) level decreased gradually until one month of age. After that it increased markedly and reached the adult level by the end of the investigation or at 6 months of age. The IgM level also continued to decrease gradually for seven days, and then increased markedly until 6 months of age, although it failed to reach the mean adult level by that time.

In two precolostral newborn foals, the serum transferrin level was 250 and 335 mg/100 ml, respectively, both of which were with in the adult range. The transferrin level showed a tendency to increase slightly after the ingestion of colostrum, without exhibiting any significant change with the advance in age.

Table 1 gives the values of IgG, IgG(T), IgM, transferrin and total protein in young and adult horse serum, together with mean value, standard deviation and range. No significant differences were present in the mean value among three groups, that is, young horses 7 months to one year of age, nonpregnant mares 4 to 27 years of age, and pregnant mares 4 to 15 years of age. The levels of IgG, IgM and total protein, however, showed a tendency to increase progressively with the advance in age, but those of IgG(T) and transferrin remained at the constant adult levels. Only one noticeable change between the nonpregnant and the pregnant mares was a slight increase in the level of IgG(T) in the latter.

Immunoelectrophoretic analysis of proteins of horse colostrum by the aid of antisera against serum protein demonstrated a number of precipitin lines, as shown in Fig. 6. When the values (mg/100 ml) for the initial colostrum (IgG: 4200, IgG(T): 2650, IgM: 1100 and transferrin (Tf): 620) were compared with 36th-hour (IgG: 1250, IgG(T): 37, IgM: 23 and Tf: 8) or 7th-day (IgG: 160, IgG(T): 24, IgG: 10 and Tf: 4) postpartum milk, all the three immunoglobulin classes and transferrin presented very high levels. Moreover, the values for 2nd-month postpartum milk were very small (IgG: 80, IgG(T): 6, IgM: 10 and Tf: 4).

**Discussion**

Studies on the fractionation of horse serum proteins in general and on the isolation of specific proteins have been performed by numerous investigators [1, 2, 4, 8, 10, 12–14, 17, 19, 21, 23, 24, 31–41]. As a result, at least six immunoglobulin classes, IgGa, IgGb, IgGc, IgG(T), IgM and IgA, have been identified in adult horse serum, as mentioned above. Sardesal and Rao [36] have also found the presence of eight antigenically distinct immunoglob-
Table 1. Levels of immunoglobulins, IgG, IgG (T) and IgM, transferrin and total protein of normal horse sera

<table>
<thead>
<tr>
<th>Group</th>
<th>Age</th>
<th>No. of animals</th>
<th>Concentrations*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IgG mg/100 ml</td>
</tr>
<tr>
<td>Young horse</td>
<td>7 months to 1 year</td>
<td>15</td>
<td>Mean 1197</td>
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<td></td>
<td></td>
<td></td>
<td>±SD 313</td>
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<td></td>
<td></td>
<td></td>
<td>Range 700–1600</td>
</tr>
<tr>
<td>Nonpregnant mares</td>
<td>4 to 27 years</td>
<td>10</td>
<td>Mean 1640</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>±SD 377</td>
</tr>
<tr>
<td>Pregnant mares</td>
<td>4 to 15 years</td>
<td>20</td>
<td>Mean 1622</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>±SD 250</td>
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<td></td>
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<td>Range 1150–2500</td>
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</tbody>
</table>

Remarks:
*: Concentration of immunoglobulins and transferrin were determined by single radial immunodiffusion and total protein by biuret method.

Immunoglobulins in horse antiserum against diphtheria and tetanus toxins. In the present study, only three major classes of immunoglobulins, IgG, IgG(T) and IgM, were isolated by DEAE cellulose chromatography and Sephadex gel filtration, while IgA could not be isolated in a pure form. A total of six precipitin lines, which consisted of five lines defined as IgGa, IgGb, IgGc, IgG(T) and IgM, and one line corresponding presumably to IgA, were detected, however, in immunoglobulin regions of adult horse serum by immuno-electrophoresis developed with antiserum against normal adult whole serum. The results of the present observation were in good agreement with those of previous studies [32, 38].

There is little information on changes in the serum proteins of the newborn foal. Polson [29] found the complete absence of the \( \gamma \)-globulin fraction and only traces of \( \beta \)-globulin at birth. Rouse [34], having carried out both immuno-electrophoresis and Oudin's quantitative method, also found that the newborn foal lacked detectable serum immunoglobulins prior to the ingestion of colostrum. In the present study, precolostral foal serum, or cord serum, was proved to be entirely deficient in immunoglobulins by less sensitive methods, such as cellulose, disc, and immuno-electrophoresis. These results are in agreement with those of the above-mentioned studies. The three classes of immunoglobulins, IgG, IgG(T) and IgM, however, were detected at a very low level by using a more sensitive method of quantitative immunodiffusion. A similar situation has also been found in swine [30] and cattle [18, 22]. At all events, it is evident that the postpartum transport of colostral immunoglobulins from mother to young is of tremendous importance for the horse, as well as swine and cattle, since there is apparently little or no transplacental transfer of immunity.

Generally, in species in which maternal immunity is transferred postnataally to young, the intestinal absorption of the immunoglobulins of colostrum is regarded as only source of immunoglobulin. Such transfer takes place across the epithelium of the small intestine of the young. It is
known that the gut of calves is permeable to antibody for 24 to 366 hours following birth [9, 28]. Bruner et al. [6] stated that the absorption of antibody by the foal ceased between 24 and 36 hours after birth. Rouse [34] also demonstrated that foals absorbed a large quantity of protein soon after sucking, and that the serum level of the three immunoglobulins, IgG, IgG(T) and IgM, reached the adult range by 12–24 hours of age. In the present study, the three immunoglobulins attained significant levels within 3 hours after the beginning of sucking and reached nearly maximal levels within 24–36 hours, declining gradually in level thereafter. It seemed reasonable to assume that the absorption of the immunoglobulins into the blood stream of the newborn foal may take place via the gut for 24 to 36 hours after birth.

A serological relationship have been established between the proteins of serum and those of milk in several animals. In the horse, Genco et al. [12] found that the colostrum contained IgGabc, IgG(T) and IgM, but little or no secretory IgA. According to Vaerman et al. [38], the equine colostrum is much richer in IgGabc, IgM and IgA, a little richer in IgG(T), and poorer in IgGc than the equine serum, whereas the equine milk contains IgA as a predominant immunoglobulin and IgM and the various IgG subclasses as minor ones. Although it was impossible to determine IgA quantitatively in the present study, the results of observation of this study were in good agreement with those of previous studies mentioned above.

The values of IgG(T) in the present study were much larger than those estimated by Rouse [34] in 55 samples collected from two- to three-year-old Thoroughbreds, whereas the IgG and IgM levels in the present study were in good agreement with those estimated by the previous author, and those by McGuire et al. [23] in ponies. Since the quantitative method by single radial immunodiffusion used in this study was the same as used by the above-mentioned investigators, this difference could not be explained readily by the variation in technique or any error in the measurement.

It is known that the levels of serum total protein and globulins, particularly immunoglobulins, can be influenced by age, breed, sex or health condition of animals and various environmental factors. In horses, Rouse [34] and McGuire et al. [23] suggested that the difference in breed and some environmental or feeding factors might influence the IgG(T) level. It has been observed that a large amount of IgG(T) was produced in horses hyper-immunized with tetanus or diphtheria toxoid [1, 36, 40].

In the present study, blood samples were obtained from 15 young horses seven months to one year of age consisting of 13 Thoroughbreds and 2 Anglo-Arabians, and 30 older horses four to 27 years of age consisting of 25 Thoroughbreds and 5 Anglo-Arabians. Furthermore, two-thirds of the adult mares were pregnant and most of the remaining one-third in lactation. It seems reasonable to assume, therefore, that the above-mentioned disparity in IgG(T) level may have resulted from various physiological variations, including the influences of the health conditions of animals and environmental factors.

Iron-binding protein called transferrin or lactoferrin have been found in the milk whey of man and various animals [3, 15, 16, 26], but there is no information on the transmission of transferrin in the horse. From their studies, Jordan and Morgan [16] pointed out that the level of iron-binding proteins was extremely high in
rabbit milk whey, and that at least transferrin and albumin were transferred from blood to colostrum. Similar studies made by Vaerman [37] on transferrin derived from canine and porcine milk indicated that significant amounts of albumin and transferrin were present only for some days after the beginning of lactation. As can be seen in the present study, more transferrin was present in colostrum than in serum in the horse, whereas much less transferrin was contained in milk whey than in serum in the mare.

It was also observed in newborn foal serum that the transferrin level rose slightly for a short time after the first ingestion of colostrum and then declined to remain rather constant within the range of normal adult level. In connection with this, it was possible to infer that transferrin and probably albumin or some other protein might be transmitted from the colostrum to the serum of the suckling foal across the intestinal wall, since these proteins rose temporarily in level in newborn foal serum after feeding. The significance of the high level of transferrin in the colostrum or its function, however, is unknown at present.

References


Explanation of Figures

Fig. 3. Electrophoretic analyses of adult horse serum (WS) and isolated antigens, IgG, IgG(T), IgM, and transferrin (Tt). A: polyacrylamide gel electrophoresis. B: immunoelectrophoresis developed with rabbit antiserum to normal adult horse whole serum. Anode is on the left.

Fig. 4. Polyacrylamide gel electrophoresis of horse serum. A: mother horse. B: precolostral newborn foal. C~H: colostrum-fed newborn foals. C: 1 hour; D: 3 hours; E: 6 hours; F: 12 hours; G: 24 hours; and H: 36 hours after the first feeding. I~M: foals. I: 3 days; J: 7 days; K: 2 months; L: 4 months; and M: 6 months of age. Anode is on the left.

Fig. 5. Immunoelectrophoresis of horse serum. A: mother horse. B: precolostral newborn foal. C~H: colostrum-fed newborn foals. C: 1 hour; D: 3 hours; E: 6 hours; F: 12 hours; G: 24 hours; and H: 36 hours after the first feeding. I~O: foals. I: 3 days; J: 7 days; K: 2 months; L: 3 months; M: 4 months; N: 5 months; and O: 6 months of age. Anode is on the right.

Fig. 6. Immunoelectrophoresis of horse serum, and colostrum and milk whey. SM: mother serum; SF1: precolostral newborn foal serum; SF2: colostrum-fed newborn foal serum 12 hours after the first feeding; M1: colostrum whey just at parturition; M2: 36th-hour postpartum milk whey; M3: 3rd-day postpartum milk whey; M4: 7th-day postpartum milk whey; and M5: 2nd-month postpartum milk whey. Anode is on the right.