

Analysis of immunoglobulin, complements and CRP levels in serum of captive northern pig-tailed macaques (*Macaca leonina*)

Xiao-Liang ZHANG^{1,2}, Wei PANG², De-Yao DENG³, Long-Bao LV⁴, Yue FENG¹, Yong-Tang ZHENG^{2,4,*}

1. Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, Yunnan 650500, China

2. Key Laboratory of Animal Models and Human Disease Mechanisms of Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China

3. Department of Clinical Laboratory, the Second Hospital of Yunnan Province, Kunming, Yunnan 650021, China

4. Kunming Primate Research Center, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China

Abstract: The northern pig-tailed macaque (NPM, *Macaca leonina*) has become a widely used animal model in biomedical research. In this study, we measured serum immunoglobulin IgG, IgM, IgA, complement C3, C4 and CRP levels in 3–11 year old captive northern pig-tailed macaques using HITACHI 7600-20 automated chemistry analyzer in order to determine the influences of age and gender on these items. The results showed that serum IgA, IgM, C3 and C4 levels were not correlated with age ($P>0.05$), while serum IgG levels increased progressively with age ($r=0.202$; $P=0.045$). Serum IgG, IgA, IgM and C3 levels were higher in females than in males ($P<0.05$). Moreover, serum C3 concentration was both positively and strongly correlated with that of C4 ($r=0.700$; $P<0.0001$). This study provides basic serum immunoglobulin and complement data of captive northern pig-tailed macaques, which may prove useful for future breeding efforts and biomedical research.

Keywords: Northern pig-tailed macaque (*Macaca leonina*); Immunoglobulin; Complement; C-reactive protein

Macaque species, including pig-tailed macaques (*Macaca nemestrina* group), rhesus macaques (*M. Mulatta*) and cynomolgus macaques (*M. fascicularis*), have been widely used in biomedical research (Yoshino et al, 2000) because of their phylogenetic proximity to humans, such as in HIV infection (Kuang et al, 2009; Lei et al, 2013; Zhu et al, 2010), chlamydial infection (Patton et al, 2001), *Campylobacter* infection (Flores et al, 1990), immunogenetics (Knapp et al, 1996), immune cell function (Li et al, 2012; Shaulov & Murali-Krishna, 2008), xenotransplantation (Watts et al, 2012), neurophysiology (Rausell et al, 1998) and cognitive behavioural studies (Macellini et al, 2010; Sussman & Ha, 2011). The taxonomic status of Macaque species have been re-evaluated, and three subspecies have been corrected into three independent species: northern pig-tailed macaques (NPMs, *M. leonina*), southern pig-tailed macaques (*M. nemestrina*) and Mentawai macaques (*M. pagensis*)

(Groves, 2001; Kuang et al, 2009).

While these studies provide critical information, to date that physiological and biochemical characteristics of captive NPMs have not been as well described as those of the other commonly-used macaques. We have previously reported the reference values of blood chemistry and hematology of NPMs (Pang et al, 2013), but little is known about the normal levels of its basic immunological parameters, such as serum immunoglobulins, complements and CRP levels, which may closely correlate with age,

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* Corresponding author, E-mail: zhengyt@mail.kiz.ac.cn

gender, breeding environment and sampling methods (Curry, 2001).

In this study, we analyzed the levels of serum IgG, IgM, IgA, C3, C4 and CRP of healthy captive NPMs with different age and gender, which will likely benefit further applications towards infectious diseases, immunology and other biomedical researches.

MATERIALS AND METHODS

Animals

One hundred NPMs used in this study were housed in Kunming Institute of Zoology, Chinese Academy of Sciences (KIZ, CAS). Animals were free of any known virus, bacteria and parasites and visually showed with no trauma, no pregnancy and no estrus. Age, gender and

body weight information were presented in Table 1. These NPMs were maintained in social groups in enclosures at room temperature and provided with a diet of fruits, monkey biscuits, and vegetables at 0800h, 1300h and 1600h, respectively. Animal care was performed according to the regulations and recommendations of the Animal Care Committee of KIZ, CAS.

Serum samples

Blood samples (1–2 mL) from each subject were collected sterilely from the saphenous vein without anesthesia, and allowed to clot at room temperature. Serum was further separated by centrifuging at 2,500 r/min, 4 °C for 20 minutes 1-hour later after bleeding and stored at –80 °C until use.

Table 1 Age, gender and body weight of NPMs

Groups	Female			Male		
	Number (<i>n</i>)	Age (years)	Body weight (kg)	Number (<i>n</i>)	Age (years)	Body weight (kg)
3–4 years old (Juveniles)	11	3.4±0.5	2.37±0.33	24	3.5±0.5	2.51±0.29
5–11 years old (Adults)	39	6.5±1.5	3.83±0.86	26	6.6±1.7	3.87±1.38
Total	50	5.8±1.9	3.51±0.98	50	5.1±2.0	3.21±1.22

Assays for serum immunoglobulins, complements and CRP

Serum specimens were delivered to Department of Clinical Laboratory, the Second Hospital of Yunnan Province, China, and then the turbidimetric immunoassay determining levels of serum IgG, IgM, IgA, C3, C4 and CRP was performed on a HITACHI 7600-020 automated chemistry analyzer using rabbit anti-human polyclonal antibodies, protein standards and reagents from Orion Corporation according to users' manuals. IgA, IgG, IgM, C3, C4 and CRP standards were diluted into known concentrations. Next, protein standards dilutions and serum specimens were measured simultaneously. Upon adding a serum sample, target antigen and polyclonal antibodies would form an immune complex that would precipitate and then increase the turbidity of the reaction solution. When light was shone on the sample, some were scattered, some were absorbed, and the rest passed through. The automated chemistry analyzer can measure the sample's light absorbance, which is positively correlated with the protein concentrations in it. Accordingly, target serum protein level could be calculated by referring to this protein's known diluted concentration series. Detailed protocols are available from the manufacturer.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 5.0. The normal distributions were tested by the Kolmogorov-Smirnov test. Spearman partial correlation coefficients were calculated by taking gender as control and taking each serum immunological parameters as dependent variables against age. When dependent variables were significantly correlated with age, then gender effects were analyzed under covariance analysis taking gender as a factor and age as a covariate. Other times, an unpaired *t*-test was used to compare the means between male and female groups. Pearson's *r*-test was used to analyze the significance in correlations between each serum immunological variables. All analyses were two-tailed, with results expressed as mean±SD, with *P*=0.05 being statistically significant.

RESULTS

Levels of serum immunoglobulins, complements and CRP

The levels of serum immunoglobulins, complements and CRP in captive NPMs from different gender and age groups are shown in Table 2. Among the total serum samples, 34 samples were showed with no detectable CRP signals, and another 34 samples displayed CRP levels less than 0.1 mg/L (data not shown).

Effects of age on the levels of serum immunoglobulins and complements

While the development, maturation and functional decline of immune system are age-related biological processes, we assessed the effects of age on the levels of serum immunoglobulins and complements in the 3–11 years old subjects. We found that the serum IgG levels were significantly increased with age ($r=0.202$; $P=0.045$), whereas, the serum IgA ($r=0.146$; $P=0.149$), IgM ($r=-0.073$; $P=0.474$), C3 ($r=-0.055$; $P=0.591$) and C4 ($r=-0.164$; $P=0.105$) levels showed no significant correlations with age (Figure 1).

Gendered levels of serum IgG, IgA, IgM, C3 and C4

Given the differences between female and male immune systems, we tested whether levels of serum IgA, IgG, IgM, C3 and C4 of females were significantly different with those of males. As shown in Figure 2, significant gender-related differences were observed in the levels of serum IgG ($P<0.001$), IgA ($P=0.001$), IgM

($P<0.001$) and C3 ($P=0.031$). All of the female serum IgA, IgG, IgM and C3 levels, especially IgM levels, were higher than those of the male. However, there was no significant gender-related difference in the serum C4 levels ($P=0.777$) between male and female groups.

Correlation analysis of serum immunoglobulins and complements

The complement system is closely correlated with immunoglobulins. Certain immunoglobulins can activate the complement system, and the activated complement system can therefore enhance the biological activities of immunoglobulins. We analyzed the correlations among serum IgA, IgG, IgM, C3 and C4 levels in NPMs and the statistical analysis showed significantly positive correlations only between the following pairs of components: IgA and IgM ($r=0.457$; $P<0.0001$), C3 and IgM ($r=0.246$; $P=0.014$), C3 and IgA ($r=0.295$; $P=0.003$), C3 and IgG ($r=0.289$; $P=0.004$), C3 and C4 ($r=0.700$; $P<0.0001$) (Figure 3).

Table 2 Levels of serum immunoglobulins and complements of NPMs

Grouping	3–4 years old (Juveniles)	5–11 years old (Adults)	Total	
IgG (g/L)	Female	17.236±2.900	19.808±3.47	19.242±3.491
	Male	16.954±2.509	17.570±2.875	17.274±2.696
	Total	17.043±2.598	18.912±3.403	18.258±3.257
	Range	12.400–22.900	10.400–30.000	10.400–30.000
IgA (g/L)	Female	0.924±0.377	1.041±0.060	1.015±0.375
	Male	0.739±0.327	0.815±0.359	0.779±0.343
	Total	0.797±0.349	0.951±0.383	0.897±0.377
	Range	0.260–1.550	0.290–2.290	0.260–2.290
IgM (g/L)	Female	2.025±0.652	1.864±0.693	1.886±0.682
	Male	1.488±0.503	1.406±0.485	1.445±0.490
	Total	1.656±0.600	1.670±0.651	1.665±0.631
	Range	0.820–3.590	0.580–4.040	0.580–4.030
C3 (g/L)	Female	1.851±0.419	1.892±0.316	1.883±0.337
	Male	1.813±0.240	1.688±0.297	1.748±0.276
	Total	1.825±0.301	1.810±0.322	1.816±0.314
	Range	1.310–2.650	1.030–2.560	1.030–2.650
C4 (g/L)	Female	0.315±0.097	0.298±0.087	0.302±0.089
	Male	0.321±0.095	0.274±0.091	0.297±0.095
	Total	0.319±0.094	0.228±0.089	0.299±0.091
	Range	0.100–0.510	0.100–0.480	0.100–0.510

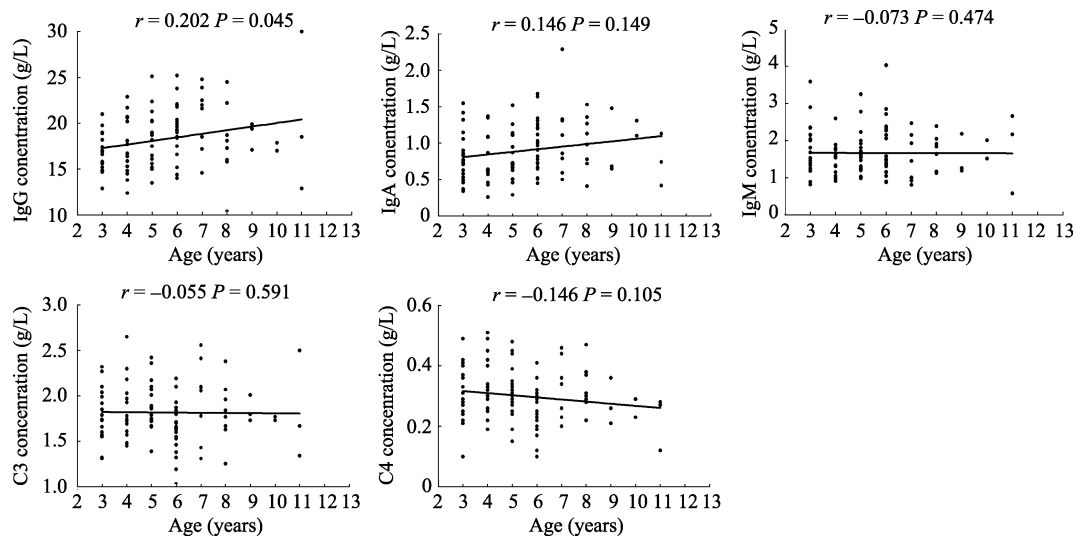


Figure 1 Effects of age on the levels of serum IgG, IgA, IgM, C3 and C4 in NPMs

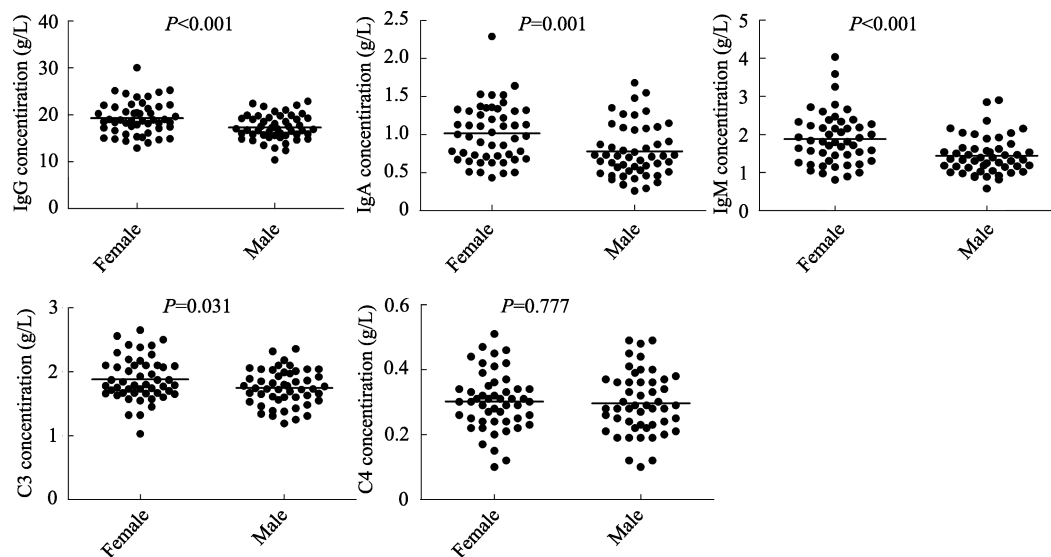


Figure 2 Effects of gender on the levels of serum IgG, IgA, IgM, C3 and C4 in NPMs

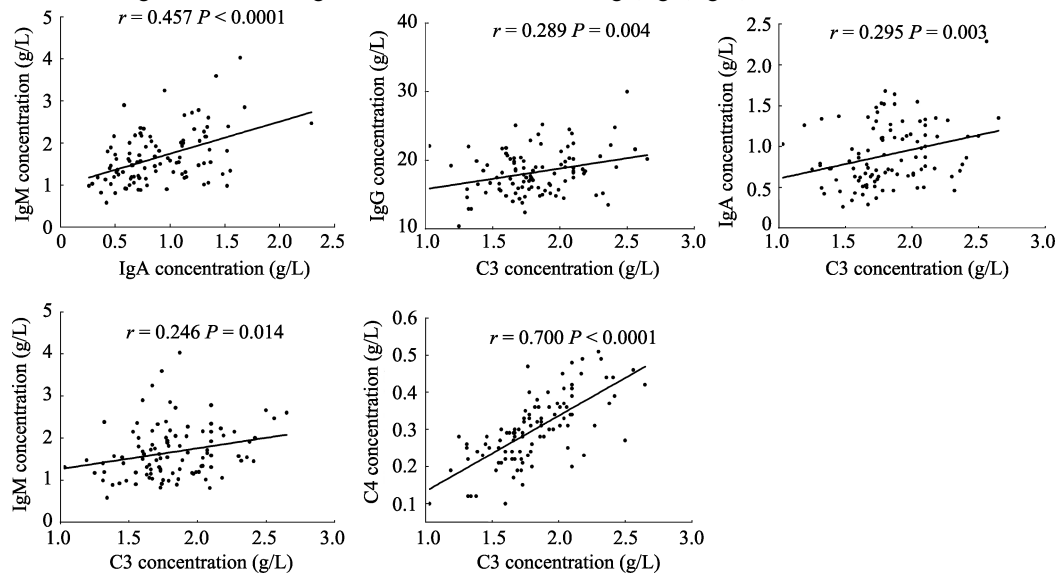


Figure 3 Correlations of serum immunoglobulins and complements in NPMs

DISCUSSION

Due to the high phylogenetic proximity between humans and non-human primates, proteins—such as immunoglobulins, complements and CRP—share a high degree of homology. Earlier studies evaluated the cross-reactivities between anti-human antibodies and non-human primate antigens and demonstrated that the homology could reach to 80%–100% (Jinbo *et al.*, 1998; Shuster *et al.*, 1969). Therefore, lots of monkey proteins have been measured with homologous antibodies of human (Axberg *et al.*, 1991; Chen *et al.*, 2006; Cheng *et al.*, 2003; Cole & Bowen, 1976; Fujimoto *et al.*, 1982; Mayer *et al.*, 1993). Moreover, in many previous studies, anti-human polyclonal antibodies have been successfully used in determining serum immunoglobulin, complement and CRP levels in rhesus macaques and/or cynomolgus macaques (Jinbo *et al.*, 1998; Socha *et al.*, 1993; Yoshino *et al.*, 2000).

Immunoglobulins play a critical role in the immediate defense against infection and its quantitative internal concentration determination is helpful in disease diagnosis, monitoring and medication. In our study, we found no effects of age (3–11 years old) on the serum IgA and IgM levels of NPMs, which was consistent with the findings in rhesus macaques and cynomolgus macaques. However, serum IgG levels increased progressively with age, which was inconsistent with previous studies on rhesus monkeys and cynomolgus macaques (Cheng *et al.*, 2003; Cole & Bowen, 1976). Such contradictory results are probably due to the interspecies differences in growth and development. The serum IgA, IgG and IgM levels of female NPMs were higher than those of males, which was in accordance with studies on other species. It has been documented that in general, females have better humoral immunity than their male counterparts (Ahmed *et al.*, 1985; Ahmed & Talal, 1990; Grossman, 1984), by showing higher immunoglobulin levels in serum, particularly IgM (Allansmith *et al.*, 1968). This gender-specific differences in serum IgA, IgG and IgM levels could mainly due to the effects of sex hormones (Ahmed *et al.*, 1985; Ahmed & Talal, 1990). NPMs were characterized with similar levels of serum IgG and IgM, but lower level of serum IgA compared with rhesus macaques and cynomolgus macaques. However, the serum IgA levels between rhesus macaques and cynomolgus macaques was similar (Cole & Bowen, 1976; Miller *et al.*, 1992; Patterson *et al.*, 1976). This phenomenon could be explained by the

perspectives of species evolution. Pig-tailed macaques were estimated to have evolutionarily differentiated from rhesus macaques and cynomolgus macaques approximately 5 million years ago (mya). However, the divergence between rhesus macaques and cynomolgus macaques occurred approximately 2.4 mya (Morales & Melnick, 1998). Additionally, blood chemistry and hematology parameters of captive populations of NPMs also have indicated that the differences between NPMs and rhesus macaques or cynomolgus macaques were greater than those between rhesus macaques and cynomolgus macaques (Pang *et al.*, 2013). As the most important and richest immunoglobulin in the mucosal immune system, the low level of IgA in the mucosal immune system may result in reduced mucosal resistance to microbial infection. In the future, it may be worth exploring and then exploiting whether or not the lower serum IgA level is related with the lower IgA of the mucosal immune system. Consequently, understanding the differences of serum and mucosal IgA levels between NPMs and other experimental non-human primates will likely benefit from its application to infectious disease studies, vaccine evaluation, as well as other fields of viral immunology.

Compared with antibodies, the complement system had an earlier origin. Complements not only act as cofactors and enhancers of antibody activities, but also have other biological functions independent of antibodies. The complement system plays an important role in innate defense against common pathogens, the modulation of inflammatory response and coagulation. For example, C3 is critical in activating the whole complement system, and C4 is the major protein of classical pathways (Kasperska-Zajac *et al.*, 2013). As such, determining the serum levels of C3 and C4 in healthy population is helpful in disease diagnosis. The levels of complements increased after delivery, so that by 3–6 months of age the mean levels of serum C3 and C4 in the infant populations are already similar to those in the normal adults (Fireman *et al.*, 1969). Our results showed that serum C3 and C4 levels did not fluctuate with age (3–11 years old), which was consistent with Cheng *et al.*'s (2003) study on rhesus macaques. While serum C3 levels of female NPMs were higher than those of males, no significant gender-specific differences were found in serum C4 levels. This phenomenon could be explained by sex hormone differences, since it has long been documented that sex hormones can markedly affect the immune system (Ahmed *et al.*, 1985; Verthelyi &

Klinman, 2000), and also influence pro-inflammatory cytokines IL-6 and IL-1 production (Angstwurm et al, 1997; Cannon & Dinarello, 1985). Moreover, evidence suggested that IL-1 and IL-6 have an enhancing effect on the production of C3, whereas neither IL-1 nor IL-6 affect the biosynthesis of C4 (Falus et al, 1990). Serum C3 levels of NPMs were close to those of rhesus macaques and cynomolgus macaques, whereas, serum C4 levels of NPMs were higher than those of rhesus macaques but lower than those of cynomolgus macaques (Cheng et al, 2003; Poskitt et al, 1974), which had indicated that non-human primates displayed marked interspecies variations regarding complements (Ellingsworth et al, 1983). Additionally, the changes in complement pathway activities were involved in the acute rejection after xenotransplantation and the pathogenesis of systemic autoimmune diseases (Chen et al, 2010; Saadi et al, 2004; Yu & Whitacre, 2004). Ultimately then, when non-human primate animal models are applied to studying human complement-related diseases, it is necessary to consider and understand the species-specific characteristics of the complement systems.

CRP is a classical acute-phase serum protein, which interacts with complements, neutrophils, monocytes, etc, and functions not only as an inflammation regulator, but also as a host defender against infection. CRP is primarily synthesized in livers and is simultaneously released into the bloodstream during acute phase response. CRP testing has been applied in differential diagnosis of infectious diseases (Du Clos & Mold, 2004), assessing cardiovascular risks (Albert et al, 2002) and monitoring autoimmune diseases progression. Previous studies reported that both the detection methods and many other factors may influence the quantifications of serum CRP levels (Zhang et al, 2011; Khera et al, 2005)). In our study, no detectable signals of CRP were found in 34 serum samples. We assume this is due to traces of serum CRP in NPMs had outreached the instrumental detection limit (0.01 mg/L), which is special designed for

human beings.

We also analyzed correlations among serum IgA, IgG, IgM, C3 and C4 levels of NPMs. As we known, complement systems, immunoglobulins, and the related components are closely interacted with each other. Serum levels of certain complement components are highly correlated with those of certain immunoglobulins (Plebani et al, 1984), and are functioned as bridges between innate and adaptive immune responses. The classic pathway is activated by immune complexes of IgG, IgM and complements, meanwhile, IgG and IgA immune complexes are acting as activators of the alternative pathways (Roach et al, 1981; Wagner & Frank, 2009). In addition, pro-inflammatory cytokines, for example IL-6, are modulators of the levels of IgG, IgM, IgA and some complements (Maes et al, 1997; Ritchie et al, 2004). Roach et al (1981) found that serum components of certain classical pathways and their alternative pathways were significantly correlated ($r > 0.537$). Yilmazer et al (2003) showed that serum C3 and C4 levels were highly correlated ($r > 0.6$, $P < 0.001$). In some diseases, the correlations between serum complement components and immunoglobulins levels was markedly altered (Gewurz et al, 1968; Kohler & Muller-Eberhard, 1969). In our study, we found significant positive correlations between the following pairs: IgA and IgM, C3 and IgM, C3 and IgA, C3 and IgG, C3 and C4, all of which are consistent with the findings in other species.

In conclusion, this study shows that serum IgA, IgM, C3 and C4 levels in NPMs were irrelevant with age, whereas, IgG levels increased progressively with age. Serum IgG, IgA, IgM and C3 levels were higher in females than in males, meanwhile, C3 concentrations were positively and closely correlated with those of C4. These basic data of captive NPMs may, in the future, promote its future and more detailed application to infectious diseases, immunology and other fields of biomedical research.

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