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Mariarosaria Marinaro°*, Anna Lucia Bellacicco, Michele Camero, Elvira Tarsitano, Maria Tempesta, Antonio Cassone°, Canio Buonavoglia

°Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy

Department of Veterinary Public Health, Università degli Studi di Bari, Bari, Italy.

*Corresponding author:

Mariarosaria Marinaro, Ph.D.
Department of Infectious, Parasitic and Immune-mediated Diseases
Istituto Superiore di Sanità
Viale Regina Elena 299
00161 Rome, Italy
mary_marinaro@libero.it
Fax +39.080.4679.843
Tel.+39.080.4679.841

Running head: caprine herpesvirus–specific IgG subclasses

Abstract

Enzyme-linked immunosorbent assays (ELISAs) were developed to detect caprine herpervirus-1 (CpHV-1)-specific IgG1 and IgG2 in sera from 43 naturally-infected goats. The analysis of the IgG subclasses showed a dual pattern of distribution in seropositive goats with a major group of animals (36 out of 43) exhibiting significantly higher levels of IgG2 over IgG1 and a minor group (7 out of 43) possessing equal levels of IgG1 and IgG2. Four goats were experimentally infected with a virulent CpHV-1 Ba.1 strain by the intranasal or the intravaginal route and the kinetics of appearance of CpHV-1-specific IgG, IgG1 and IgG2 in the serum were studied. Two weeks following infection, both IgG1 and IgG2 levels increased although convalescent sera (i.e., collected five to eight weeks post-infection) showed a clear prevalence of the IgG2 subclass. To determine the contribution of the different IgG subclasses to herpesvirus immunity, serum neutralization (SN) assays were performed in both naturally and experimentally infected goats. The kinetics of SN showed that neutralization activity was mainly associated to the IgG1 subclass and this was also confirmed in naturally infected goats. The results are discussed from the standpoint that the profile of the IgG subclasses is instrumental to study immune responses to CpHV-1 and that vaccination strategies may benefit from this information.

Keywords: CpHV-1; IgG subclass; viral neutralization; IgG; goat immunity

1. INTRODUCTION

Caprine herpesvirus 1 (CpHV-1) belongs to the *Herpesvirales* order, *Herpesviridae* family, *Alphaherpesvirinae* subfamily, *Varicellovirus* genus (Davison et al., 2009) and causes two different syndromes depending on the age of the animal at the time of infection. In adult goats, the infection is often subclinical or it results in vulvovaginitis, balanoposthitis or spontaneous abortion (Grewal and Wells, 1986; Saito et al., 1974; Tarigan et al., 1987; Williams et al., 1997). In one- or two-week old kids, CpHV-1 is responsible for a systemic disease characterized by high morbidity and mortality, and ulcerative and necrotic lesions throughout the enteric tract (Mettler et al., 1979; Van der Lugt and Randles, 1993). CpHV-1 enters the animals via the genital or the respiratory tract and establishes latent infection in the sacral and trigeminal ganglia (Tempesta et al., 1999b). In natural infection, it can reactivate during the mating season probably as a result of the stress associated to hormonal changes (Tempesta et al., 1998) while in experimental infection, reactivation can be achieved following administration of high doses of dexamethasone (Buonavoglia et al., 1996). CpHV-1 infection is distributed worldwide although it is responsible for major economical losses in Mediterranean countries (Thiry et al., 2006). The CpHV-1 shares many biological similarities with human herpesvirus 2 (HHV-2) and although many reports have shown that mice could be used to study HHV-2 infection (reviewed in Ferenczy, 2009) others have shown that the CpHV-1/goat is a useful model to study HHV-2 infection in humans (Tempesta et al., 1999a; 1999b; 2000; 2007c). Thus, implementation of the studies on CpHV-1 will also provide benefit to human medicine where vaccines against HHV-2 are urgently needed (Ferenczy, 2007). Although some data has accumulated on the pathogenesis and latency of CpHV-1 infection, there is limited

information on the immune responses to CpHV-1 (Tempesta et al., 2005; 2007b) and no information is available on IgG subclasses.

Cattle have three transcriptionally active genes encoding three IgG subclasses (Rabbani et al., 1997), namely IgG1, IgG2, and IgG3; sheep and goats also seem to have the same genes (Butler, 1998; Micusan and Borduas, 1977) but the lack of commercially available antibodies specific to IgG3 dampens the elucidation of the functional role of the third IgG subclass in these ruminants.

Caprine IgG1 and IgG2 responses have been studied by western blot analysis (employing cross-reactive anti-bovine IgG subclass antibodies) following genetic vaccination with a caprine arthritis-encephalitis virus envelope gene (Cheevers et al., 2000) and in *Chlamydophila psittaci* infection where it is important to determine the profile of the IgG subclass in order to distinguish clinical inapparent infections from overt disease (Schmeer et al., 1987). The aim of the present study was to evaluate the distribution of CpHV-1-specific IgG1 and IgG2 in caprine herpesvirus infection by developing CpHV-1-specific ELISAs. In addition, the contribution of the IgG subclasses to the neutralization activity of sera from naturally and experimentally infected goats was also studied.

2. MATERIALS AND METHODS

2.1. Animals

To select the naturally-infected goats, 63 goats of mixed breed, age (range 1-4 years) and sex (10 males, 53 females) were screened for serum IgG by using a CpHV-1-specific ELISA. Among the 63 animals screened, there were 20 seronegatives and 43 seropositives; the latter were used for the subsequent evaluation of serum CpHV-1-specific IgG subclasses. For the experimental infection, four additional goats (females, age 3-4 years) were employed. All the animals included in the study were free of caprine arthritis-encephalitis virus. Blood samples were aseptically obtained from the jugular vein and serum was collected by centrifugation at 2000 rpm for 10 min (Beckman microfuge, Fullerton, USA). Serum samples were stored at –20° C until tested.

2.2. Virus

The Ba.1 strain of CpHV-1 was used to experimentally infect the animals or to prepare the antigen for the ELISA (Buonavoglia et al., 1996). The virus was propagated by infecting Madin Darby Bovine Kidney cells (MDBK; ATCC-LGC Standards, Milan, Italy) grown on Dulbecco-Minimal Essential Medium (D-MEM; Lonza, Walkersville, USA) supplemented with 10% foetal calf serum (FCS; Lonza). The viral titer was $10^{7.00}$ 50% tissue culture infectious doses ($TCDI_{50}$)/50µl. The viral suspension was tested for bacterial or fungal contamination.

2.3. Experimental infections

For the intravaginal infection, two goats received 4 ml of CpHV-1 Ba.1 strain ($10^{7.00}$ $TCDI_{50}$/50µl) in the vagina. For the intranasal infection, two goats received 2 ml per nostril of the same viral suspension as described above. Goats were kept under observation for two months and examined daily for general and clinical signs, including body temperature, as described previously (Tempesta et al., 2000; 2007a; 2007b; 2007c). At the indicated time points, goats were bled and sera were collected and stored at -20° C until tested by ELISA and neutralization assays.

2.4. Elisa for serum IgG, IgG1 and IgG2

To measure CpHV-1-specific IgG responses, an assay in use in our laboratory was employed (unpublished). In particular, 96-well polystyrene plates (Nunc, Roskilde, Denmark) were coated with 100 µl/well of CpHV-1 (total protein content, 25 µg/ml) in carbonated buffer (15mM Na_2CO_3, 35 nM $NaHCO_3$, [pH 9,6]) and incubated overnight at 4° C on a shaker. After blocking the wells with 200 µl of 2 % gelatin/PBS (Sigma, Milan, Italy) for 2 hours at 37°C, individual serum samples diluted 1:100 in 0.05% Tween 20/PBS (PBS/T) were added to duplicated wells and incubated overnight at 4° C. After washing, HRP-conjugated rabbit anti-goat IgG (Bethyl, Montgomery, USA) diluted 1:1000 in PBS/T were added to the wells and incubated for 2 hours at 37° C. After final washings and addition of ABTS, the colorimetric reaction was measured at 405 nm with an ELISA plate reader (Biorad, Hercules, USA). The O.D. values were recorded and individual readings

were reported by subtracting the O.D. values of negative controls (i.e., negative serum samples) to the individual O.D. values. All serum samples were run under identical conditions and, where possible, on the same microtiter plate. The O.D of negative control sera or background wells (without serum) was 0.030-0.050.

To measure IgG subclasses, a protocol similar to that employed for total IgG was employed with some modifications: a) serum samples were diluted 1:10 and incubated overnight at 4° C; b) HRP-conjugated sheep anti-bovine IgG1 (diluted 1:100 in PBS/T) or HRP-conjugated sheep anti-bovine IgG2 (diluted 1:50 in PBS/T) were employed as detection antibodies (Bethyl); c) the latter detection antibodies were incubated overnight at 4° C. The O.D. values (read at 405 nm) were recorded and individual readings were reported by subtracting the O.D. values of negative controls (i.e., negative serum samples) to the individual O.D. values. All serum samples were run under identical conditions and, where possible, on the same microtiter plate. The O.D of negative control sera or background wells (without serum) was 0.040-0.050 for IgG1 and 0.045-0.055 for IgG2. Preliminary experiments established that sheep anti-bovine IgG1 or IgG2 cross-reacted with goat IgG subclasses. In addition, we performed serum dilution curves with several sera exhibiting a different ratio IgG1:IgG2; over a large serum dilution range, the curves generated with the anti-IgG1 and the anti-IgG2 were parallel to each other (not shown).The distribution of IgG subclasses was not affected by breed, age or sex.

2.5. SN assays

Serum neutralization assays were performed as described elsewhere (Tempesta et al., 2000). Briefly, sera were heat inactivated at 56° C for 30 min and serial two-fold dilutions

were mixed with 100 $TCID_{50}$ of CpHV-1 Ba.1 strain in 96 well flat bottom plates (NUNC). Plates were incubated at room temperature for 90 min and then 20000 MDBK cells were added to each well. Following three days of incubation at 37°C with 5% CO_2, the endpoint titers were determined as the highest serum dilution which neutralized the cytopathic effect of the virus.

2.6. Statistical analyses

Comparison of data was performed by using Student's *t*-test or ANOVA (when variance between groups was different) and is indicated in the figure legends. A *p* value less than 0.05 was considered significant.

3. RESULTS

3.1. Distribution of CpHV-1-specific serum IgG1 and IgG2 in naturally infected goats.

To select the seropositive animals, 63 goats were initially screened for CpHV-1-specific serum IgG by using an ELISA. Figure 1 shows the CpHV-1-specific IgG levels in the 63 goats. Forty-three seropositive animals were identified and were subsequently employed for the evaluation of the IgG subclasses, while the 20 seronegative goats were excluded from the analysis. When the 43 positive sera were subjected to the CpHV-1-specific IgG1 and IgG2 ELISAs, the distribution of the IgG1 and IgG2 segregated into two different profiles. Figure 2A shows the distribution of CpHV-1-specific IgG1 and IgG2 in a major group of animals, i.e. 36 goats out of 43, referred to as Group 1. These animals exhibited IgG2 levels significantly higher than IgG1 ($p < 0.05$). A minor group of animals, i.e. 7 goats out of 43, possessed a different distribution of IgG1 and IgG2 than Group 1. This minor group was referred to as Group 2, and included all the animals possessing equal levels of IgG1 and IgG2 in their sera (Fig. 2B). Since the differences between Group 1 and Group 2 seemed not solely qualitative but also quantitative, a statistical comparison was made between Group 1 and Group 2 for IgG1, IgG2 and IgG levels. The animals belonging to Group 2 exhibited significantly higher levels of IgG1 ($p < 0.05$), IgG2 ($p < 0.05$) and IgG ($p < 0.05$) than Group 1.

3.2. Experimental mucosal infection with a virulent Ba.1 strain of CpHV-1 results in dominant IgG2 responses.

A study was then conducted to determine whether infecting the animals by different mucosal routes or sampling the sera at different time points (post-primary infection) could result in a different distribution of IgG subclasses. Four goats were experimentally infected, two by the intravaginal (Figs. 3-5A) and two by the intranasal (Figs. 3-5B) route, and the kinetics of appearance of total IgG and IgG subclasses in the serum was measured at different time points post-infection. CpHV-1-specific IgG were detected two weeks post-infection in all animals and they continued to increase during the following two to four weeks and reached a maximum level five to eight weeks post-infection (Fig. 3). A parallel analysis was made for CpHV-1-specific IgG subclasses (Fig. 4) and it was observed that both IgG1 and IgG2 were detected in comparable amounts two weeks post-infection and continued to show a similar increase for four weeks post-infection. However, at later time points, while IgG1 continued to be detected essentially at the same level, IgG2 levels rose significantly, becoming the dominant subclass in convalescent sera (i.e., five to eight weeks post-infection). Thus, following primary infection, the final distribution of IgG subclasses was not influenced by the route of infection; on the other hand, sampling at different time points determined a different profile of IgG subclasses.

3.3. Contribution of the IgG1 subclass to SN activity

To study the role of the IgG subclasses to CpHV-1 immunity, the SN endpoint titers were determined in both naturally and experimentally infected animals. An initial comparison was made between the SN titers obtained with sera from Group 1 and Group 2 of naturally infected goats. Although there was a slight increase in Group 2 (mean SN titer 87 ± 64 for Group 2 and 54 ± 48 for Group 1) the differences between Group 1 and 2 were not

statistically different. However, with the limited number of animals in Group 2 (n=7) and the high variability of SN titers in Group 1 (along with variable IgG2 and IgG levels) it was not possible to obtain a proper statistical comparison. Therefore, among the animals of Group 1, the few animals exhibiting the same levels of IgG2 as Group 2 but possessing a significantly lower amount of IgG1 than Group 2 (this subgroup was referred to as Subgroup 1) were selected and employed for a direct comparison with Group 2 (Table I). A direct statistical comparison was thus possible since the role of IgG1 in SN could be evaluated with no interference by IgG2. Table I shows that SN titers and IgG1 levels in Subgroup 1 were lower than those of Group 2. Finally, the kinetics of the SN endpoint titers was also detemined by using sera from the four experimentally infected goats. Figure 5 shows that, while high SN titers were detected two weeks post infection, they remained relatively stable in the following five to eight weeks post-infection i.e., at the time when the IgG2 subclass increased significantly (Fig. 4).

4. DISCUSSION

The paucity of data on caprine IgG subclasses, prompted us to develop CpHV-1-specific ELISAs to measure the distribution of IgG1 and IgG2 in CpHV-1 infected goats. Here we show that natural and experimental infection resulted in dominant IgG2 responses with minor production of IgG1 antibodies.

The distribution of antigen-specific IgG subclasses can mirror the secretory activity of Th1- and Th2-type lymphocytes in both humans and animals (Mosmann et al., 1986; 1989; Finkelman et al., 1990; Parronchi et al., 1991; Stavnezer, 1996; Liblau et al., 1995; Staats at al., 1994; Marinaro et al., 1998). Thus, in a Th1 dominant setting, the production of IFN-gamma may promote IgG1 and IgG3 responses in humans (Gregorek et al., 2000; Hussain et al., 1999; Pène et al., 2004; Widhe et al., 1998), IgG2a in mice (Snapper and Paul, 1987) and IgG2 in cattle (Estes et al., 1994). On the other hand, Th2-type lymphocytes, secreting IL-4, support the production of IgG4 in humans (Couper et al., 1998; Hillman et al., 2004; Ishizaka et al., 1995; Lundgren et al., 1989) and IgG1 in mice and cattle (Snapper et al., 1988; Estes et al., 1995). Since immune responses in goats resembles those observed in cattle (Butler, 1983; 1998), the prevalence of CpHV-1-specific IgG2 antibodies reported here could probably reflect the production of IFN-gamma during herpesvirus infection.

The dominance of IgG2 over IgG1 observed in the majority of infected goats is consistent with data reported with other species infected by herpesviruses. In fact, humoral responses to HSV in humans are prevalently sustained by IgG1 and IgG3 (Gilljam et al.,1985; Mathiesen et al., 1988; Sundqvist et al., 1984) and mice produce high levels of IgG2a antibodies in response to HSV/viral infection (Coutelier et al., 1987; Ishizaka et al., 1995; McKendall and Woo, 1988). The distribution of antigen-specific IgG subclasses is also

informative of the immune defenses mounted by the host (Butler, 1998; Finkelman et al., 1990; Micusan and Borduas, 1977; Stavnezer, 1996). In this regard, goat IgG2, as well as human IgG1/IgG3 and mouse IgG2a, bind Fc receptors on macrophages and PMN (Anderson and Abraham, 1980; Finkelman et al., 1990; Micusan and Borduas, 1977) and can act as opsonin or promote ADCC with both mechanisms contributing to antibody-mediated herpesvirus clearance (Butler, 1998; Ferenczy, 2007; Ishizaka et al., 1995; Kohl, 1991; Mathiesen et al., 1988; Micusan and Borduas, 1977). Thus, it could be speculated that regardless of the species under investigation, the distribution of IgG subclasses following primary herpesvirus infection is the result of the dominant role played by IFN-gamma which drives the synthesis of the most appropriate IgG subclass.

It should be underlined that IgG4 are also produced in response to primary herpesvirus infection in humans although HSV-seropositive humans generally exhibit low IgG4 levels which is in agreement with the low IgG1 levels observed in both naturally infected goats (Group 1) and experimentally infected goats. Nonetheless, the IgG4 subclass is increased in patients with nonprimary or recurrent HSV infections (Coleman et al., 1985; Hashido and Kawana, 1997; Ljungman et al., 1988) or after massive antigenic stimulation (Aalberse et al., 1983). Thus, either recurrent or nonprimary infections could have contributed to the high levels of IgG1 (and IgG) found in the minor group of naturally infected goats (Group 2). It also remains to be investigated whether the IgG2 responses can be boosted by a subsequent CpHV-1 infection since Group 2 exhibited higher IgG2 responses as well.

It is remarkable that the SN activity was mainly associated to the IgG1 subclass. Indeed, in naturally infected goats the SN titer decreased with decreasing IgG1 levels. In addition, in experimentally infected goats, the comparison of the kinetics of SN with that of IgG

subclasses suggested that both subclasses could account for the observed SN activity in the first two weeks post infection; however, since the IgG2 levels continued to increase five to eight weeks post infection without causing a parallel increase in the SN endpoint titers, we speculate that IgG2 may not be directed to neutralizing epitopes. It would be necessary to purify IgG1 or IgG2 from immune sera in order to study, in vitro and in vivo, the neutralizing ability of each purified IgG subclass and their specificities.

Our data would help to design effective vaccines since vaccination strategies (which are aimed at preventing infection) should take into account the fact that IgG1 responses could be more relevant for CpHV-1 neutralization and, therefore, adjuvants and delivery systems able to potentiate IgG1 (and IgG2) in goats must be preferred (Simms et al., 2002). In agreement with this, our previous reports have shown that reactivation of infection in naturally infected goats is more frequent in animals with low SN titers (Tempesta et al., 1998) and that high SN titers are associated to the production of IgG specific to gB, gC, and gD, glycoproteins which are involved in viral binding and fusion/penetration (Tempesta et al., 2005). Finally, IgG1 is the major antibody present in the colostrum and milk of bovines and goats (Butler, 1983; 1998; Micusan and Borduas, 1976; 1977), thus appropriate vaccination of adults goats would confer better passive immunity to newborns where CpHV-1 infection is often lethal.

The findings reported here have dual implications. They help dissect the caprine immune responses to herpesvirus but they are also instrumental to design preventative or therapeutic tools to be used in goats as well as in humans. In this context, the CpHV-1/goat is an excellent model to study HHV-2 infection in humans. We have now shown that the profile of the IgG subclasses in CpHV-1 infected goats mimicks that seen in HHV-2 infected

humans. Although mice have been largely used to study HHV-2 infection they may not be an ideal model (Ferenczy, 2007) while goats may offer selective advantages over rodents (Hein and Griebel, 2003; Hugo Johansson et al., 1985); however, major efforts have to be made to overcome the lack of species specific reagents and the absence of detailed information on the immune system of this veterinary species. The development of assays to study CpHV-1-specific cellular and humoral immune responses in goats may significantly impact the field of CpHV-1/HHV-2 research. This is therefore the focus of our current investigation.

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Legends to Figures

Figure 1

CpHV-1-specific serum IgG in naturally infected goats. Sixty-three goat sera were screened by using a CpHV-1-specific IgG ELISA. There were 43 seropositive animals which were then used to analyze the CpHV-1-specific IgG subclasses while the 20 seronegative goats were not included in the subsequent study. Reported data are mean + SD.

Figure 2

Distribution of CpHV-1-specific serum IgG1 and IgG2 in the 43 naturally infected goats. IgG subclass levels were determined by using CpHV-1-specific IgG1 and IgG2 ELISAs. The distribution of IgG subclasses in the 43 seropositive goats segregated into two different profiles. A major group of animals (named Group 1 and consisting of 36 out of 43 goats) exhibited significantly higher IgG2 levels than IgG1 (Fig. 2A; $p < 0.05$ by the Student's t test). A minor group of seropositve goats (named Group 2 and consisting of 7 out of 43 goats) exhibited equal levels of IgG1 and IgG2 (B).

Figure 3

Kinetics of CpHV-1-specific serum IgG responses following experimental CpHV-1 infection. Four goats were experimentally infected with a virulent CpHV-1 Ba.1 strain; two goats were infected by the intravaginal route (A) and two goats were infected by the intranasal route (B). At the indicated time points post-infection, sera were taken and CpHV-1-specific IgG levels were measured by ELISA. Reported data are mean + SD.

Figure 4

Kinetics of CpHV-1-specific serum IgG1 and IgG2 responses following experimental CpHV-1 infection. Four goats were experimentally infected with a virulent CpHV-1 Ba.1 strain; two goats were infected by the intravaginal route (A) and two goats were infected by the intranasal route (B). At the indicated time points post-infection, sera were taken and CpHV-1-specific IgG1 and IgG2 levels were measured by ELISA. Reported data are mean + SD.

Figure 5

Kinetics of SN endpoint titers following experimental CpHV-1 infection. Four goats were experimentally infected with a virulent CpHV-1 Ba.1 strain; two goats were infected by the intravaginal route (A) and two goats were infected by the intranasal route (B). At the indicated time points post-infection, sera were taken and SN assays were perfomed. The SN endpoint titers were determined as the highest serum dilution neutralizing the cytophatic effect of the virus in vitro. Reported data are mean + SD.

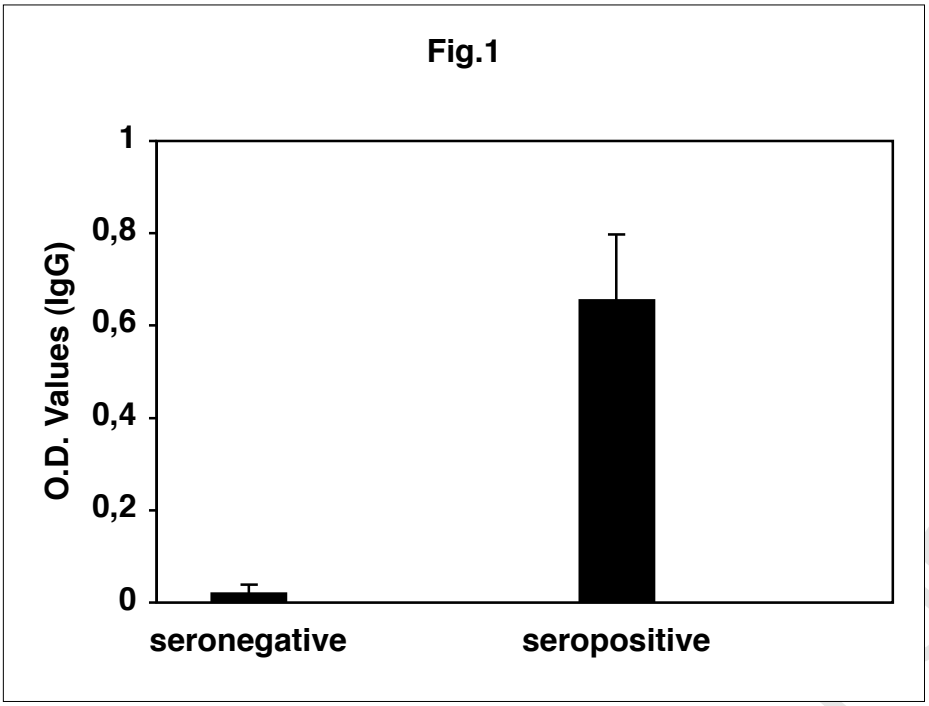

Fig. 2A

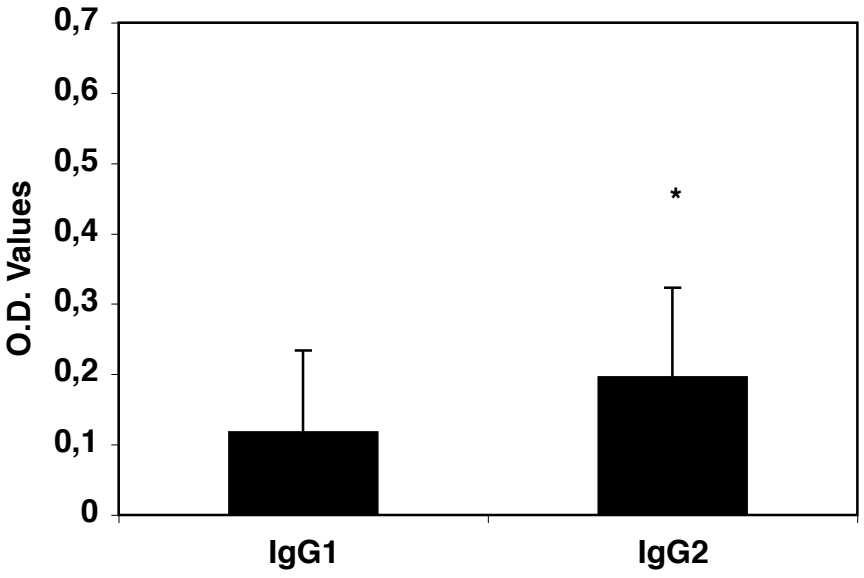

Fig. 2B

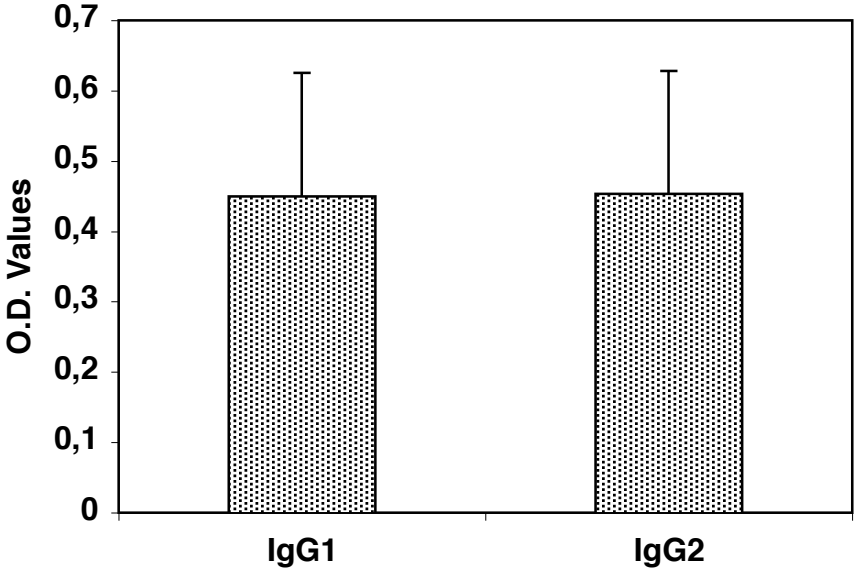

Fig. 3A

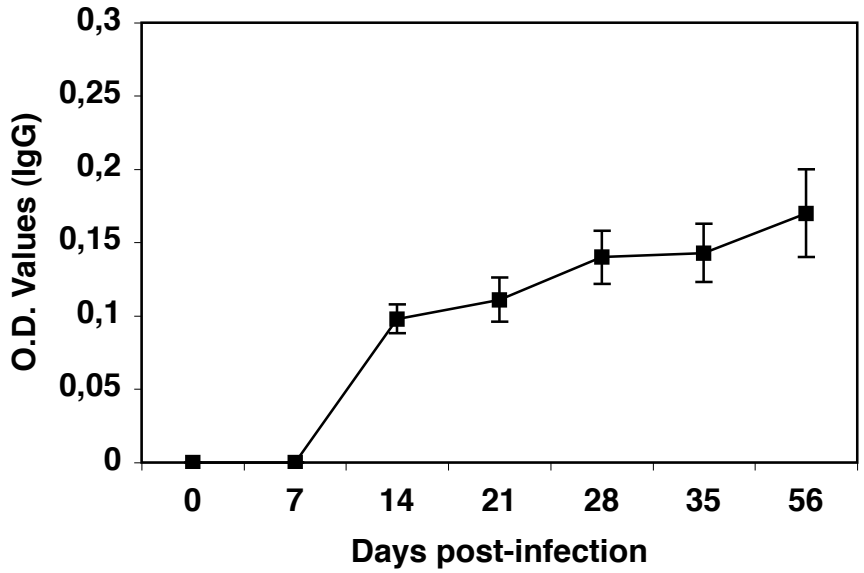

Fig. 3B

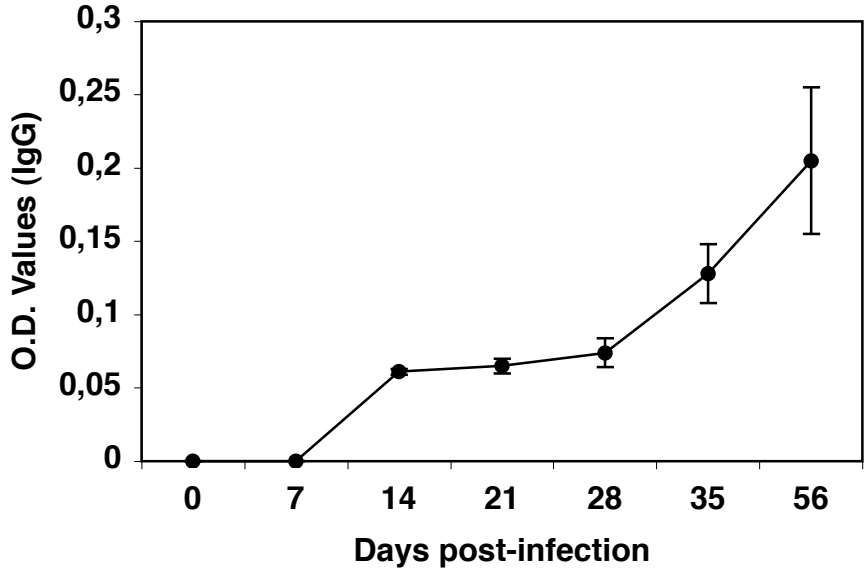

Fig. 4A

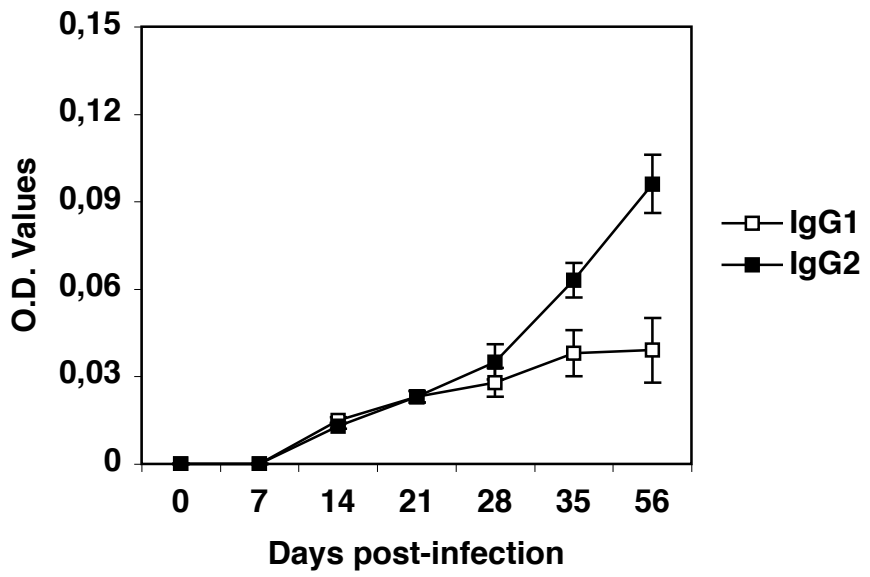

Fig. 4B

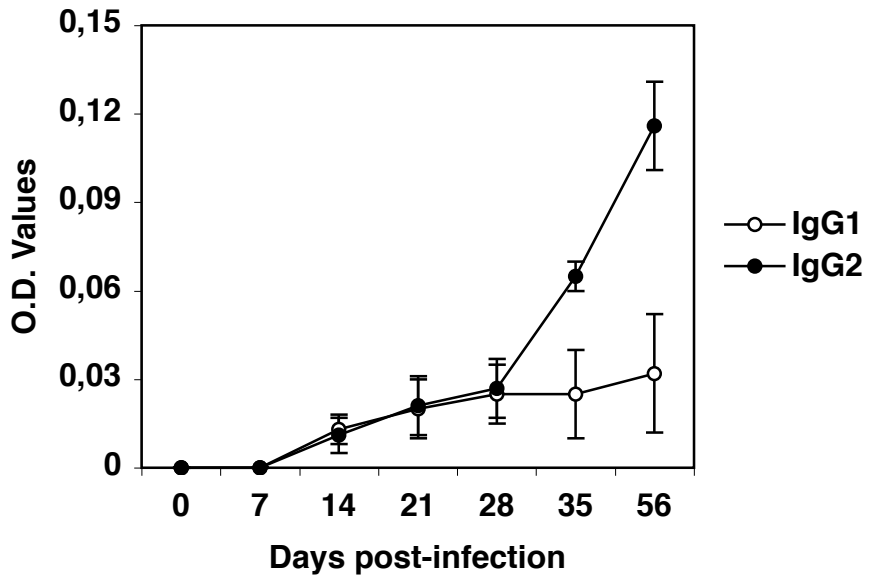

Fig. 5A

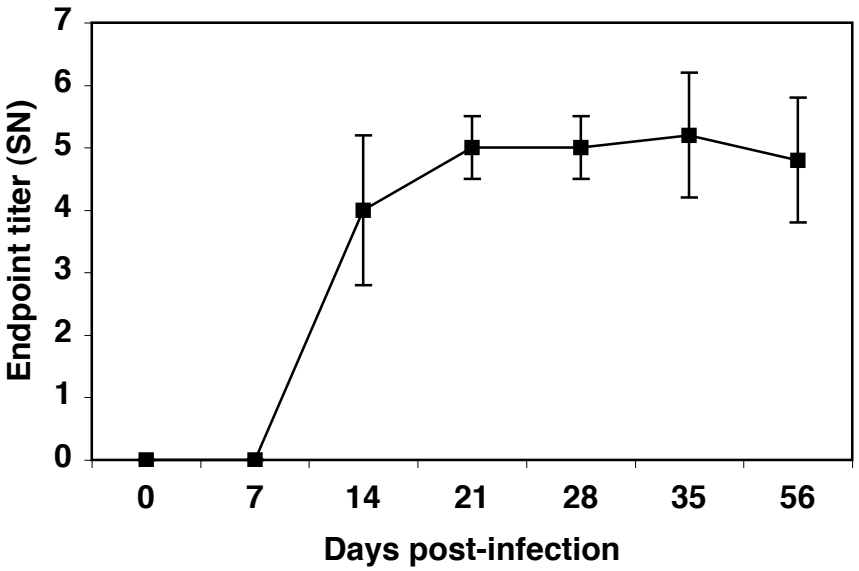

Fig. 5B

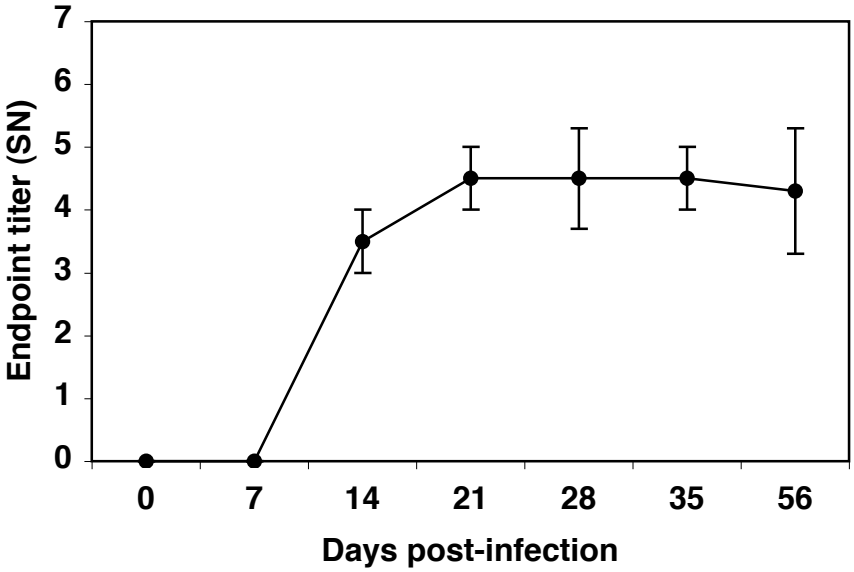

Table I **CpHV-1-specific IgG1 are mainly associated to the SN activity in naturally infected goats**

	SN titer +/- SD	IgG1 +/- SD[a]	IgG2 +/- SD[a]
SubGroup 1[#]	29 +/- 16	0.216 +/- 0.136	0.334 +/- 0.142
Group 2	87 +/- 64 *	0.450 +/- 0.175 *	0.453 +/- 0.175

[a] ELISA O.D. Value

[#] Among the 36 goats of Group 1, a subgroup of 6 goats was selected according to the following criteria: IgG2 levels were not significantly different from Group 2; IgG1 levels significantly lower than those of Group 2. The SN titers of the Subgroup 1 and Group 2 were then statistically compared.

*p value < 0.05 Group 2 *vs* SubGroup 1 by the ANOVA test