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## Neutralization of different echovirus serotypes by individual lots of intravenous immunoglobulin

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9 **Neutralization of different echovirus serotypes by individual lots of**  
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11 **intravenous immunoglobulin**  
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41 With the exception of Hans D. Ochs, all authors are employees of Baxter BioScience.  
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55 Running head: Echovirus neutralization by IVIG  
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## 1 Abstract

2  
3 Replacement therapy using intravenous immunoglobulin (IVIG) preparations in people  
4 with antibody deficiencies is effective in preventing the majority of common bacterial and viral  
5 infections, yet echovirus break-through infections have occurred. Currently, only limited  
6 information on neutralization capacity variability of individual IVIG lots against the different  
7 echovirus serotypes is available.

8 Infectivity assays were established for the most prevalent echovirus serotypes (E 9, E 11,  
9 E 13 and E 30) circulating in the United States (US) and the European Union (EU). The  
10 echovirus serotype specific neutralization titers of 41 IVIG lots manufactured from either whole  
11 blood (Recovered) or collected by apheresis (Source) and from either the US or EU, were  
12 determined.

13 Significantly higher ( $P < 0.0001$ ) neutralization titers against E 11 and E 30 were found in  
14 IVIG lots manufactured from US Source plasma compared to US Recovered plasma.  
15 Geographically, IVIG lots made from US plasma contained significantly ( $P < 0.0001$ ) higher  
16 neutralization titers against E 9 and E 11 than lots manufactured from EU plasma, whereas lots  
17 made from EU plasma showed significantly higher neutralization of E 30.

18 To conclude, IVIG lots differ in their neutralizing antibody content against different  
19 echovirus serotypes, depending on plasma collection practices and geographic origin.

20 Based on these results, an informed choice in selecting IVIG lots with the highest available  
21 neutralization titer against the specific echovirus serotype would seem to be beneficial during  
22 treatment of break-through infections.

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1 **Keywords**

2 Intravenous immunoglobulin, human - IVIG; echoviruses; virus neutralization; antibody  
3 functionality; replacement therapy

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## 1 Introduction

2 Echoviruses belong to the human Enterovirus B group (family *Picornaviridae*) and  
3 45 serotypes can be distinguished [CDC, 2006]. They are distributed worldwide and echovirus  
4 infections have been associated with diverse clinical outcomes, ranging from asymptomatic  
5 infection to minor febrile illness or persistent and severe, fatal conditions [Abzug, 2004]. More  
6 recently, an involvement of echovirus in the development of chronic diseases such as  
7 type 1 diabetes has been suggested [Viskari et al., 2004]. It has been estimated that echovirus is  
8 responsible for 10 to 15 million symptomatic infections in the US each year [CDC, 2006].  
9 Clinically severe manifestations of echovirus infections are reported most frequently in two  
10 patient groups: (i) infants and young children (up to 4 years of age), accounting for approximately  
11 60% of all echovirus infections [CDC, 2006], who are prone to develop severe echovirus  
12 infection with significant morbidity from sepsis, meningoencephalitis or myocarditis  
13 [Abzug et al., 1993; Abzug et al., 1995; Abzug, 2004; McKinney et al., 1987] and, (ii) people  
14 with antibody deficiency, particularly those with X-linked agammaglobulinemia, are at high risk  
15 to develop severe and / or chronic echovirus infections, particularly  
16 meningoencephalitis [McKinney, 1987].

17 While no specific therapy for echovirus infection is available, current clinical treatment  
18 regimen still include pleconaril [American Academy of Pediatrics, 2006; Abzug et al., 2004,  
19 Desmond et al., 2006]. Also, as antibodies are known to play an important role in the defense  
20 against enteroviral infections [Abzug et al., 1995], treatment of echovirus infection with  
21 intravenous immunoglobulin (IVIG) preparations has been suggested as beneficial [Abzug et  
22 al., 1995; Erlendsson et al., 1985; Galama et al., 2000; Johnston and Overall, 1989; Mease et  
23 al., 1981; Nagington, 1982; Nagington et al., 1983]. IVIG is a therapeutic preparation that  
24 contains the pooled antibodies from thousands of healthy plasma donors. Two different plasma

1 collection practices are utilized, where 'Recovered' plasma is collected from whole blood  
2 donations and 'Source' plasma is obtained from donors by apheresis. Previous work has shown  
3 that administration of IVIG with high echovirus antibody content improves clinical outcome  
4 [Abzug et al., 1995; Erlendsson et al., 1985; Galama et al., 1997; Galama et al., 2000; Johnston  
5 and Overall, 1989; McKinney et al., 1987; Mease et al., 1981; Nagington, 1982; Nagington et  
6 al., 1983] or protect those at risk of infection, especially during the echovirus season from June to  
7 October [CDC, 2006]. Although some of these studies have reported echovirus antibody titers for  
8 IVIG preparations and corresponding clinical efficacy, the specific results obtained are of limited  
9 value for current clinical use, since none of the products analyzed are available anymore [Abzug  
10 et al., 1995].

11 A number of echovirus serotypes have been associated with chronic enteroviral  
12 meningoencephalitis in agammaglobulinemic patients and are therefore of greatest concern for  
13 the Primary Immune Deficiency community (an estimated 250,000 children and adults in the US  
14 alone) [Boyle and Buckley, 2007]. The most frequent echovirus serotypes producing chronic  
15 enteroviral meningoencephalitis are: E 11 > E 9 > E 30 and > E 13 [McKinney, 1987]. In  
16 addition, E 13 has recently caused re-occurring severe disease in children with primary immune  
17 deficiency (R. Schiff, pers. communication). Echovirus serotypes show distinct epidemiologic  
18 characteristics and temporal patterns of circulation [Strikas et al., 1986]. Throughout the last  
19 decades E 9, E 11 and E 30 were reported most frequently in the general US [CDC, 2006] and  
20 EU [Ortner et al., 2009; RKI, 2010] populations, whereas E 13 has been circulating in the EU for  
21 some time but has only recently been associated with disease in the US [Mullins et al., 2004].  
22 Previous efforts to link echovirus occurrence in the general population with echovirus antibody  
23 content of IVIG were not successful [Galama, 2000]. Other studies that investigated IVIG  
24 antibody specificities for different pathogens in IVIG lots suggested that antibody content varies

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3 1 according to plasma donor age [Modrof et al., 2008], vaccination status [Audet et al., 2006;  
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5 2 Farcet et al., 2010] and virus epidemiology [Planitzer et al., 2007]. Because anecdotal evidence  
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7 3 suggests that understanding the echovirus antibody variation in IVIG lots could be of clinical  
8  
9 4 relevance for the treatment of persons with primary immune deficiency [Abzug et al., 1995;  
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11 5 Erlendsson et al., 1985; Galama et al., 2000; Johnston and Overall, 1989; Mease et al., 1981;  
12  
13 6 Nagington, 1982; Nagington et al., 1983], neutralization capacity of 41 IVIG lots against  
14  
15 7 echovirus serotypes E 9, E 11, E 30 and E 13 were determined. These serotypes are amongst the  
16  
17 8 most relevant for individuals with primary immune deficiency who are at increased risk of severe  
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19 9 systemic disease. This study presents the echovirus neutralization capacity of currently available  
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21 10 IVIG preparations and highlights important differences between IVIG lots manufactured from  
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23 11 plasma collected in different geographies and from different plasma donor population segments.  
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## 1 **Methods**

### 2 **Viruses and cells**

3 Virus isolates were obtained from the American Tissue Culture Collection (ATCC; E 9  
4 strain 'Hill', ATCC VR-39; E 11 strain 'Gregory', ATCC VR-41; E 13 strain 'Del Carmen',  
5 ATCC VR-43 and E 30 strain 'Bastianni', ATCC VR-322) and propagated and titrated on Vero  
6 cells (European Collection of Cell Cultures, ECACC no. 84113001).

### 8 **IVIG preparations**

9 KIOVIG™ (Baxter AG, Vienna, Austria) and Gammagard Liquid™ (Baxter Healthcare Corp.,  
10 Westlake Village, United States), are 10% liquid IVIG preparations produced by the same  
11 manufacturing process. The licensed shelf life for these products is two years when stored at  
12 2-8°C and the IgG subclass distribution is within the physiological range, i.e. IgG1 ≥ 56.9%,  
13 IgG2 ≥ 26.6%, IgG3 ≥ 3.4% and IgG4 ≥ 1.7% [Teschner et al., 2007].

14 A total of 41 IVIG lots were analyzed in 2008. 23 lots were made from US plasma (12 lots from  
15 Recovered and 11 lots from Source plasma) and 18 lots fractionated from EU plasma (2 lots from  
16 Recovered and 16 lots from Source plasma). The dates of manufacture ranged from  
17 December 2006 - November 2007 for US and from May 2006 – July 2007 for EU plasma derived  
18 IVIG lots. The limited number of IVIG lots manufactured from EU Recovered plasma did not  
19 allow a differential analysis between EU Source and Recovered plasma.

20

## 1 Titration of echovirus infectivity and determination of echovirus neutralization titers

2 Neutralization assays were performed at least in duplicate by two different operators and each  
3 assay included internal controls for the verification of virus suspension infectivity. Equal volumes  
4 containing 2,000 tissue culture infectious doses<sub>50</sub> (TCID<sub>50</sub>) echovirus and serial twofold dilutions  
5 of IVIG were mixed, incubated for one hour at room temperature and eightfold replicates per  
6 dilution were transferred to a microtiter plate containing a Vero cell monolayer. After 7 days of  
7 incubation at 37°C, cells were assessed for the presence of a cytopathic effect. The echovirus  
8 neutralization titer, expressed as the test article dilution that resulted in 50% virus  
9 neutralization (NT<sub>50</sub>), was calculated from the number of negative wells and the initial sample  
10 dilution according to the method of Reed and Muench [Reed and Muench, 1938]. Virus  
11 concentrations were calculated according to the Poisson distribution and expressed as  
12 log<sub>10</sub> TCID<sub>50</sub> per mL [Planitzer et al., 2009].

## 14 Statistical analysis

15 Statistical analysis was done using GraphPad Prism v5.0 software (San Diego, CA, USA).  
16 Differences in IVIG antibody content were analyzed using the Student's t-test  
17 (unpaired, two-tailed) and comparison of variance was done using the F-test.

## 1 **Results**

### 2 **Echovirus neutralization titers of IVIG lots derived from Recovered versus Source plasma** 3 **collected in the US**

4 There was a significant difference in neutralization titers for E 11 and E 30 (Fig. 1). IVIG lots  
5 made from Source plasma had significantly higher ( $P < 0.0001$ ) NT<sub>50</sub> values for E 11  
6 (mean  $\pm$  SEM, N; Source:  $180 \pm 7$ , 11; Recovered:  $135 \pm 5$ , 12) and E 30 (mean  $\pm$  SEM, N;  
7 Source:  $611 \pm 17$ , 11; Recovered:  $422 \pm 17$ , 12), as compared to lots derived from Recovered  
8 plasma. The NT<sub>50</sub> values were approximately 3-fold higher for E 30 than for E 11 and the  
9 difference between mean NT<sub>50</sub> values of Recovered versus Source plasma derived IVIG lots was  
10 similar within the same echovirus serotype, i.e. Source NT<sub>50</sub> values were approx. 40% higher than  
11 Recovered NT<sub>50</sub> values, for E 11 as well as for E 30. No significant difference in neutralization  
12 titers for E 9 ( $P = 0.48$ ) and E 13 ( $P = 0.48$ ) was observed between Recovered and Source plasma  
13 derived IVIG lots (Fig. 1).

14 Significant variation in NT<sub>50</sub> values obtained for IVIG against E 9 ( $P = 0.02$ ) and E 13 ( $P = 0.003$ )  
15 was seen.

### 17 **Echovirus neutralization titers of IVIG lots derived from US or EU plasma**

18 Significant differences in echovirus neutralization were observed for IVIG lots derived from US  
19 or EU plasma, for E 9 ( $P = 0.0002$ ), E 11 ( $P < 0.0001$ ) and E 30 ( $P = 0.0008$ ) (Fig. 2). IVIG lots  
20 derived from US plasma had significantly higher NT<sub>50</sub> values for E 9 and E 11, whereas  
21 EU plasma derived IVIG had significantly higher neutralization capacity for E 30. For E 13, no  
22 difference ( $P = 0.05$ ) in neutralization was observed between the IVIG lots tested (Fig. 2).

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4 1           There was significant variability in echovirus NT<sub>50</sub> values for E 9 ( $P = 0.01$ ),  
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6 2 E 13 ( $P = 0.01$ ) and E 30 ( $P = 0.01$ ) within US plasma derived IVIG lots.

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8 3           When the results were analyzed according to echovirus serotype and irrespective of  
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10 4 plasma geographic origin and collection practices, the highest NT<sub>50</sub> values were obtained for  
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12 5 E 30 (mean  $\pm$  SEM, N; 559  $\pm$  16, 41), followed by E 9 (mean  $\pm$  SEM, N; 285  $\pm$  13, 41),  
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14 6 E 11 (mean  $\pm$  SEM, N; 138  $\pm$  5, 41) and E 13 (mean  $\pm$  SEM, N; 55  $\pm$  3, 41).  
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5 2 **Discussion**  
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8 3 Even though IVIG lots are manufactured from the pooled plasma donations of several thousand  
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10 4 donors, there is still a certain level of variation in antibody content between individual lots of  
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12 5 IVIG [Lejtenyi and Mazer, 2008]. Previous studies have shown that antibody specificities in  
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14 6 IVIG depend on the plasma donor population segment from which the plasma was  
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16 7 collected [Modrof et al., 2008], their vaccination status [Audet et al., 2006; Farcet et al., 2010] or  
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18 8 virus epidemiology [Planitzer et al., 2009]. These variations are linked to plasma collection  
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20 9 practices, i.e. whole blood versus apheresis donations (Fig.1), as well as geographic origin of the  
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22 10 plasma, i.e. US versus EU (Fig.2) [Galama et al., 1997].  
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26 11 To date, only four studies have evaluated the neutralizing antibody levels against the most  
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28 12 common echovirus serotypes and reported highly variable levels in different IVIG  
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30 13 products [Abzug et al., 1995; Galama et al., 1997; Galama et al., 2000; Mease et al., 1981]. This  
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32 14 variability in reported antibody levels is, at least partially, explained by the use of different virus  
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34 15 neutralization assay formats as well as different IVIG products, which are produced using  
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36 16 different manufacturing procedures. Of note, these investigations were done prior to 2000 and  
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38 17 included a limited number of IVIG lots no longer available, thus no information on echovirus  
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40 18 antibody content in current IVIG preparations is available.  
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43 19 As it has been suggested that this information might be of vital importance to patients suffering  
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45 20 from severe echovirus infections [Abzug et al., 1995; Erlendsson et al., 1985;  
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47 21 Galama et al., 2000; Johnston and Overall, 1989; Mease et al., 1981; Nagington, 1982;  
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49 22 Nagington et al., 1983], echovirus serotype specific antibody content in 41 IVIG lots,  
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51 23 manufactured from plasma of different geographic and demographic origin, were determined.  
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3 1 The statistically higher E 11 and E 30 neutralization titers observed for Source plasma as  
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5 2 compared to Recovered plasma-derived IVIG lots produced by the same manufacturing  
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7 3 procedure during the same period of time were surprising, as earlier studies had revealed the  
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9 4 exact opposite, i.e. higher measles virus [Audet et al., 2006] and parvovirus B19 (B19V) [Modrof  
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11 5 et al., 2008] antibody titers in Recovered plasma-derived IVIG preparations. In both these cases,  
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13 6 however, the generally older age of Recovered plasma donors (approx. two thirds of Source  
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15 7 plasma donors are below 30 years of age, whereas the same proportion of Recovered plasma  
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17 8 donors are older) has been shown to result in higher virus antibody titers. In the case of measles,  
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19 9 higher titers are elicited after wild-type virus infection as opposed to exposure through a vaccine  
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21 10 [Audet et al., 2006], and for B19V, higher prevalence of antibody-positive donors is found in  
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23 11 advanced age groups [Modrof et al., 2008]. For echoviruses, however, exposure occurs uniformly  
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25 12 by infection with wild-type virus as no vaccine exists, but the likelihood of infection is greater for  
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27 13 infants and young children [CDC, 2006]. Of note, the overall level of circulation increased since  
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29 14 1975 for E 30 and since 1979 for E 11 [CDC, 2006] and thus titers can be expected to be highest  
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31 15 for younger donors, and then declining during adolescence of donors towards the average age of  
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33 16 Recovered plasma donors. However, with the limited information on the epidemiological  
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35 17 occurrence of those viruses prior to 1970, the association of significantly higher titers with the  
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37 18 younger age of Source plasma donors remains speculative.

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39 19 As IVIG products reflect the immune status of a population subset, a certain degree of  
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41 20 correlation should exist between echovirus incidence rates and echovirus neutralization capacity  
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43 21 in individual lots of IVIG. A significantly greater E 9 antibody content in  
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45 22 US plasma derived IVIG correlates well with an approximately 6-fold higher incidence of E 9  
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47 23 infections in the US [CDC, 2006] as compared to the EU [Ortner et al., 2009; RKI, 2010]. While  
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49 24 the incidence rates reported for E 11 are similar for the US [CDC, 2006] and EU  
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3 1 [Ortner et al., 2009; RKI, 2010], results outlined in the presented study suggest that this echovirus  
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5 2 serotype is more prevalent in the US, as evidenced by a significantly greater E 11 antibody  
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7 3 content in US plasma derived IVIG. The relative infrequent occurrence of E 13 in the  
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9 4 EU [RKI, 2010] and the US [CDC, 2006] is reflected by a low NT<sub>50</sub> observed in IVIG  
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11 5 preparations from both geographic areas. The high incidence of E 30 reported from the EU  
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13 6 [RKI, 2010] correlates well with the results obtained. However, the high antibody titers detected  
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15 7 for E 30 in US plasma do not correlate well with E 30 incidence rates reported by the National  
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17 8 Enterovirus Surveillance System (NESS), which indicate that E 30 is the third most prevalent  
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19 9 echovirus serotype in the US, after E 9 and E 11 [CDC, 2006]. The E 30 antibody content  
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21 10 detected in US IVIG was by far the highest for the echovirus serotypes included in this study,  
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23 11 which may suggest that E 30 infections occur more frequently in the US than reported by the  
24  
25 12 NESS, possibly reflecting underreporting due to the generally milder illness associated with this  
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27 13 serotype [CDC, 2006], or even asymptomatic infections.

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29 14 The presented results indicate that the surveyed echovirus serotypes occur at similar  
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31 15 frequencies in both geographies, i.e. E 30 > E 9 > E 11 > E 13, and that, with the exception of  
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33 16 E 30, the echovirus serotype specific antibody titers were always higher in US plasma derived  
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35 17 IVIG. These results, in conjunction with the information gained from the analysis of Recovered  
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37 18 versus Source plasma derived IVIG lots, are of importance to health care professionals who are  
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39 19 treating patients with severe echovirus infection. By choosing an IVIG lot with the highest  
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41 20 possible echovirus serotype specific antibody titer, it is possible to increase the amount of  
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43 21 administered echovirus neutralizing antibodies by up to 3-fold, thereby maximizing the possible  
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45 22 therapeutic benefit, as has previously been shown [Abzug et al., 1995; Erlendsson et al., 1985;  
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47 23 Galama et al., 2000; Johnston and Overall, 1989; Mease et al., 1981; Nagington, 1982;  
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49 24 Nagington et al., 1983]. Based on the findings of the presented surveillance study, a selection of  
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3 1 IVIG lots for substitution therapy during rare episodes of echovirus infection may be guided by  
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5 2 the recommendation given in Table I. As a certain level of variability in echovirus NT<sub>50</sub> titers was  
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7 3 already detected in this study, the echovirus serotype-specific neutralization capacity of IVIG lots  
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9 4 needs to be evaluated at regular intervals, in order to follow future trends in echovirus antibody  
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11 5 prevalence and to identify any shifts that could be relevant to substitution therapy.  
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3 **1 Conclusions**  
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5 2 The neutralization capacity of individual lots of IVIG against the most prevalent echovirus  
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8 3 serotypes varies up to threefold. These differences are linked to plasma collection practices, i.e.  
9  
10 4 whole blood versus apheresis donations, as well as geographic origin of the plasma, i.e. US  
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12 5 versus EU.  
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15 6 Despite IVIG substitution therapy, rare cases of infection by different echovirus serotypes occur  
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17 7 in people with primary antibody deficiencies. Knowledge of the echovirus serotype neutralization  
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19 8 titers of individual IVIG lots would enable targeted selection of higher titer lots against a specific  
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21 9 echovirus serotype for substitution therapy during an infection episode.  
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16 7 acknowledged.  
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For Peer Review

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5 2 Table I. Selection of IVIG lots for substitution therapy during echovirus infection episodes  
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	EU - IVIG		US - IVIG	
			Source	Recovered
E 9			x	x*
E 11			x	
E 13	x		x*	x
E 30	x		x*	

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21 3 \*NT<sub>50</sub> values varied significantly within the group  
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23 4 EU: European Union, E: echovirus, NT<sub>50</sub>: 50% virus neutralization; IVIG: Intravenous  
24 5 immunoglobulin, US: United States  
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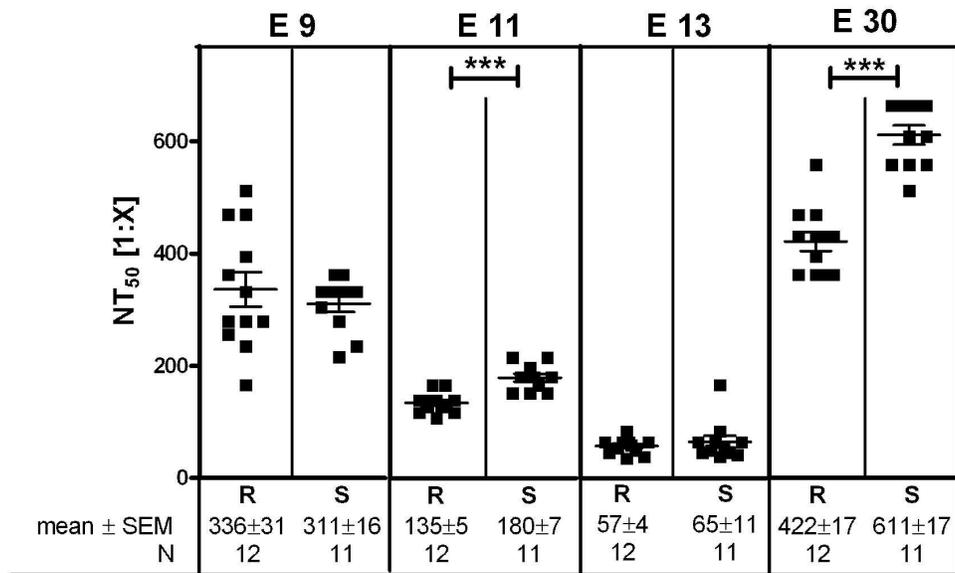
2 **Figure legends**

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4 Fig. 1. Echovirus neutralization titers 50% (NT<sub>50</sub>) of individual IVIG lots manufactured from  
5 US plasma were determined and analyzed separately by plasma collection practice,  
6 i.e. Recovered (R) from whole blood donations or Source (S) collected by apheresis. Error bars  
7 indicate standard error of the means; asterisks indicate significant difference in mean titers  
8 ( $P < 0.001$ ). N = number of IVIG lots tested.

9 Fig. 2. Echovirus neutralization titers 50% (NT<sub>50</sub>) of individual IVIG lots manufactured from  
10 plasma collected in the European Union (EU) or the United States (US). Error bars indicate  
11 standard error of the means; asterisks indicate significant difference in mean titers ( $P < 0.0001$ ).  
12 N = number of IVIG lots tested.

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Fig. 1. Echovirus neutralization titers 50% (NT50) of individual IVIG lots manufactured from US plasma were determined and analyzed separately by plasma collection practice, i.e. Recovered (R) from whole blood donations or Source (S) collected by apheresis. Error bars indicate standard error of the means; asterisks indicate significant difference in mean titers ( $P < 0.001$ ). N = number of IVIG lots tested.

149x96mm (300 x 300 DPI)

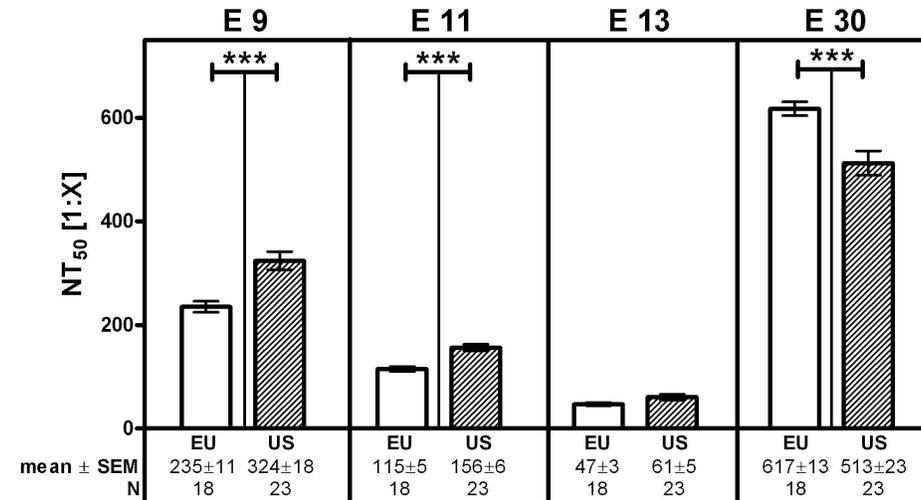


Fig. 2. Echovirus neutralization titers 50% (NT50) of individual IVIG lots manufactured from plasma collected in the European Union (EU) or the United States (US). Error bars indicate standard error of the means; asterisks indicate significant difference in mean titers ( $P < 0.0001$ ). N = number of IVIG lots tested.

213x125mm (300 x 300 DPI)