Defining thrombocyte and leukocyte reference intervals for neonatal calves (10-28 days of age) in a BNP affected population

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Abstract

Bovine neonatal pancytopenia (BNP) is a disease described in several European countries. The disease affects neonatal calves and is characterised by petechiae, cutaneous bleedings and melena, resulting in a case mortality of 90%. Haematology shows thrombocytopenia, leukocytopenia and anaemia. Pathology findings include pale mucous membranes, petechiae, ecchymoses through the whole body, thereby histology of the bone marrow shows a panmyelopthisis. To confirm case diagnosis reference intervals for thrombocytes and leukocytes are needed, however there is not much known about these reference values for neonatal calves. The current study sampled 361 control calves aged between 10 and 28 days for haematological analysis. There were no age effects observed between different age categories with regard to amount of thrombocyte and leukocytes ($P>0.05$). To define reference intervals the formula 'mean ± 1.96*standard deviation' was used. The results of the present study were used to define reference intervals for thrombocytes and leukocytes for neonatal calves in a BNP affected population.

Keywords: Bovine neonatal pancytopenia, reference interval, neonatal calf, thrombocytes and leukocytes

Introduction

Since 2007 a new syndrome of haemorrhagic diathesis in calves was observed. Especially in Germany and surrounding countries this disorder was observed (Pardon et al., 2010; Bastian et al., 2011; Witt et al., 2011). Clinical symptoms developed 10 to 20 days post partum and consist of petechiae, cutaneous bleedings and melena (Bastian et al., 2011). After the first symptoms, 90% of the calves die within five days as a result of blood loss or secondary infections (Bastian et al., 2011; Pardon et al., 2011). Post mortem examination showed pale mucous membranes, petechiae and ecchymoses through the whole body (Witt et al., 2011). Thereby, aplasia of the bone marrow in the form of a panmyelopthisis was observed (Witt et al., 2011). Haematological analysis showed that there is a thrombocytopenia, leukocytopenia and anaemia (Pardon et al., 2010; Bastian et al., 2011).

Clinical symptoms and pathology findings were associated with a novel disease which had several names; bleeding calf syndrome or blood sweating (Friedrich et al., 2011). Nevertheless, the syndrome would be termed bovine neonatal pancytopenia (BNP) (Ballingall et al., 2011). An immune mediated disorder is the cause of this syndrome (Bastian et al., 2011; Bridger et al., 2011). Bridger et al. (2011) and Friedrich et al. (2011) reported that BNP is induced after colostrum intake from dams which had a BNP calf in the past; alloreactive antibodies cause thrombocytopenia and leukocytopenia.

For confirmation of diagnosis it is helpful to have reference intervals for calves. However, reference intervals for calves generally differ greatly from adult cattle (Witt et al., 2011). Thereby, there is not much known about reference intervals for calves and in particular for the age group around 10-20 days.

The aim of the present study was to determine reference intervals for thrombocytes and leukocytes in a population of newborn calves; 10-28 days of age.

Materials and methods

Animals

In a multi-country epidemiological investigation of BNP in the Netherlands, clinical affected calves were blood sampled from January 2011 to December 2011. Cases were determined by several criteria; aged between 0-28 days, clinical symptoms, leukocytes and thrombocytes below predefined levels. The inclusion criteria for leukocytes was set at $<5.0*10^9$/L and for thrombocytes at $<150*10^9$/L (A. Smolenaars, personal communication). In addition to leukocytopenia and thrombocytopenia, diagnosis could be confirmed with bone marrow pathology. In combination with blood collecting of cases, blood samples of up to a maximum of four control calves per case were collected. Those controls originated from the same farm as the cases. Control calves had to be aged 10-28 days and clinically healthy for farmer and veterinarian, however, if no calves in this age category were present younger or older calves were sampled for this purpose. Blood collection was combined with a questionnaire to obtain additional information about colostrum, medication, illness, etc. for each calf, case or control.

Samples and lab analysis

Blood samples were collected by the project team or by the local veterinarian and were taken from the jugular vein. Samples were harvested directly into an ethylenediaminetetraacetic acid (EDTA) tube and were send to GD Animal Health Service, Deventer, the Netherlands. EDTA was chosen as anticoagulant, because it has no influence on the leukocyte and thrombocytes count (George et al., 2010). The average interval between sampling and analysing was 24 hours. Haematology was performed with an automatic analyzer (Cell-Dyn 3700; Abbott Laboratories, Abbot Park, Illinois, U.S.A.) and was used to determine leukocyte and thrombocyte counts.

Statistical method

Data on thrombocyte and leukocyte levels were analyzed in SPSS (version 16, IBM SPSS Data Collection). For definition of reference intervals the following formula was used;

'Mean ± 1.96 * standard deviation'

Data were analyzed for normal distribution and were log transformed if this was not the case. Data were log transformed to base 10, which is a known manner to normalize data (Petrie and Watson, 2006). The log formula that has been used for definition of reference interval was;

'$10^{\log}$ (mean) ± 1.96* $10^{\log}$ (standard deviation)'

To define thrombocyte and leukocyte reference intervals. Control calves with an age of 10-28 days were used. In this research two methods were applied to define reference intervals. After excluding calves for method one and two, the age category was divided in six subcategories; 10-12, 13-15, 16-18, 19-21, 22-24 and 25-28 days of age to control the possible presence of age effects. Age effects were examined using ANOVA. When age has no effect at all selected control calves were used for defining reference intervals.

The purpose of the first method was to obtain a healthy population for defining reference intervals. To obtain this, controls without a history of clinical illness or medication were used. Controls that became cases after sampling or which were doubtful controls i.e. $<150*10^9$/L thrombocytes and/or $<5.0*10^9$/L leukocytes, were excluded from analysis. Thereby controls which had been ill or had been medicated were also excluded from analysis.

The aim of method two is to obtain reference intervals data driven; all control calves aged between 10-28 days were ranked for thrombocytes and leukocytes. The controls which belong to the upper 2.5 percent and lower 2.5 percent for thrombocytes and/or the upper 2.5 percent and lower 2.5 leukocytes were all excluded, and have not been used for defining reference intervals.

Results

Animals

A total of 674 calves were included in the Dutch questionnaires of the multi-country epidemiological investigation of BNP, consisting of 143 case calves and 531 control calves. Out of the 531 control calves 361 control calves were included in this study, because of the 10-28 days age category.

Method one

After excluding control calves for method one 286 calves remained for analysis. 13 calves were excluded because they were doubtful controls or became a BNP case after sampling and 57 were excluded because they were coded with some illness or had been medicated. From the resulting 291 control calves 5 calves were removed because of missing values for thrombocytes and leukocytes. Thrombocyte and leukocytes counts were not normally distributed ($P<0.05$), therefore they were log transformed and checked again for normal distribution. Log leukocytes count were normally distributed ($P>0.05$) in contrast to log thrombocytes count ($P<0.05$). There were no age effects between sub age categories for both log thrombocytes count and log leukocytes count ($P>0.05$). All 286 control calves were used for definition of the reference interval; results of the analysis are shown in table 1. The exact reference interval determined using this method was 367.7-1547.7*10^9/L for thrombocytes and 4.64-19.20*10^9/L for leukocytes.

Method two

In total 30 control calves had values within the upper or lower 2.5 percent of the thrombocytes and/or the leukocytes count were excluded from analysis. A total of 324 control calves remained for analysis, because of missing values of 7 calves. Thrombocytes and leukocytes count were checked for normal distribution. Thrombocytes count was normally distributed ($P>0.05$) in contrast to the leukocytes count ($P<0.05$) and therefore the leukocytes count was log transformed, resulting in a normal distribution ($P>0.05$). Figure 1 and figure 2 show thrombocytes and leukocytes count in relation to age categories. Thrombocytes count and log leukocytes count were tested for age effects, though no significant effect ($P>0.05$) was found. Therefore all 324 calves were used for definition of the reference interval of thrombocytes and leukocytes. Table 1 shows the results of method two. The exact reference interval which had been calculated for thrombocytes was 302.0-1249.4*10^9/L and 4.97-18.04*10^9/L was found for leukocytes.

Discussion

Design

Within the design of the multi-country epidemiological investigation in the Netherlands only calves from 10-28 days of age were included. However, this predefined age range is maybe not correct. Clinical symptoms develop 10 to 20 days post partum (Bastian et al., 2011), so in this age category of 10-28 days there were controls which were subclinical cases and some of these even developed into cases after sampling. The presence of these subclinical cases influences the data as the lower limit for both thrombocytes and leukocytes decreases, because of thrombocytopenia and leukocytopenia. It is probably better to sample calves in the age range of 20-28 days of age, because the chance on subclinical cases will be excluded. Nevertheless in our data this did not influence results.

Methods

Method one is a doubtful method. Calves which had been ill or had been medicated were excluded along with controls which became cases after sampling or were doubtful controls. The included calves were clinically healthy, although in every population there could be some underlying infections or disorders. This allows this method to be biased. The calves could be sub clinically ill and so on; they do not represent the healthy population were this method is based on. On the other hand this method does represent a standard population, because in every population there are calves which are sub clinically ill.

The only assumption that has been made, in method two, is that in a population there are always individuals which are distinct from the rest of the population. It was assumed that in this population also some outlying calves were present, therefore the upper and lower 2.5 percent for thrombocytes and for leukocytes were excluded. The sampled controls were clinically healthy for farmer and veterinarian, but within the included controls there were probably underlying disorders or illnesses. The control calves could come from farms with clinically affected BNP calves which is not a random sample of the cattle herds. There is a substantial probability that the control calves could have lower levels of thrombocytes and leukocytes, because they originated from a population with thrombocytopenia and leukocytopenia. On the other hand, controls were sampled when they were clinically healthy and had no symptoms of BNP. Indeed, when in method two, 13 calves which became case after sampling or were doubtful controls were first excluded, followed by the same procedure, the lower limit of the reference interval for thrombocytes became $430*10^9$/L in contrast to $300*10^9$/L for the original method and for leukocytes the lower limit increased from $5.0*10^9$/L to $5.3*10^9$/L.

Optimal method

Based on the obtained data from GD Animal Health Service, is method two the best, because method two is data driven and no assumptions have been made regarding to a general population, except for the assumption that distinct calves with outlying blood values are always present. Nevertheless, data may not be correct because the sampled controls were aged between 10-28 days. Bastian et al. (2011) reports that clinical symptoms occur between 10 and 20 days of age, so this dataset does not represent a population which is necessary for obtaining reference intervals; it represents a population with possible BNP cases. It would be better to analyse the data, using calves aged from 20-28 days, following the same procedure as in method two. However, appropriate strategies for defining thrombocyte and leukocyte

reference intervals include sampling analysis of ad random calves within a predefined reference range for a year from ad random farms to exclude variables that might influence on the determination of reference intervals. Nevertheless, the proposed intervals for this data set with these two methods will be the intervals obtained in method two; $300*10^9$/L - $1250*10^9$/L and $5.0*10^9$/L - $18.0*10^9$/L for thrombocytes and leukocytes, respectively.

Variation in mean thrombocyte and mean leukocyte counts due to age

Currently there are no thrombocyte and leukocyte reference intervals for calves aged between 10-28 days, but for adult cows there are published reference intervals; 100-$800*10^9$/L for thrombocytes and 4.0-$12.0*10^9$/L for leukocytes (Knowles et al., 2000; Mohri et al., 2007; George et al., 2010). Figures 3 and 4 show the mean, including the box, of the thrombocytes and leukocytes count together with the reference intervals for adult cattle. The mean of the leukocyte and thrombocytes count for method one and method two are quite high in comparison to the adult reference interval, especially for thrombocytes were the box lays for a part outside the reference interval.

It was expected that after one week of age there was no increase in thrombocyte count. This is in agreement with other publications (Knowles et al., 2000; Brun-Hansen et al., 2006; Mohri et al., 2007). They report that in the first week of life the thrombocytes count raises to the top of the reference interval for adult cattle. After the first week Knowles et al. (2000) and Brun-Hansen et al. (2006) reported that thrombocytes count rises above the reference interval. In this study the mean of the thrombocytes count also rises above the upper limit of the reference interval after two weeks of age and decreases after three weeks of age. Nevertheless, there were no age effects between the sub age categories. Mohri et al. (2007) reported that the thrombocytes count was within the reference interval for adult cows after a week of age. Brun-Hansen et al. (2006) reported that after 19-21 weeks the thrombocytes count decreases within the reference interval for adult cattle. This is not seen in this study; there is a slight decrease as can be seen in figure 1, but at week 3 of age it already is within the reference interval for adult cows.

Mohri et al. (2007) and Brun-Hansen et al. (2006) reported that leukocytes were within the reference interval 10-28 days post partum, this is in agreement with this study; the course of the leukocytes is shown in figure 2. In contrary Knowles et al. (2000) reported that the leukocytes were between 6 and 20 days of age above the reference interval for adult cattle.

Multi-country epidemiological investigation of BNP

In an unpublished study calves in a clinic were repeatedly blood sampled up to 14 days of age to obtain reference intervals for thrombocytes by calves. The mean at 10 days after birth was $757*10^9$/L and at 14 days of age $747*10^9$/L (B.J.G. Roelofs, unpublished data). In comparison to figure 1 the mean of thrombocytes, at those two time points, is slightly higher than the results of the unpublished data.

Comparing the proposed lower limits from method two, $300*10^9$/L thrombocytes and $5.0*10^9$/L leukocytes, with the predefined inclusion criteria for BNP cases in the multi-country epidemiological investigation of BNP concludes that those predefined criteria for thrombocytes and leukocytes are well chosen. When the definition for control calves is proposed according to the proposed reference interval for leukocytes and thrombocytes a group remains which is not definable as case nor as control; $>150*10^9$/L and $<300*10^9$/L thrombocytes. Those calves have no clinical symptoms of BNP so this group could be subclinical BNP cases. This study proposes therefore that cases should have clinical symptoms and should satisfy the predefined inclusion criteria. Control calves should satisfy

the reference intervals which are proposed in this study; table 1. The rest group will be called subclinical, but only when they have thrombocytes between $150*10^9$/L and $300*10^9$/L and leukocytes below $5.0*10^9$/L. Calves which only have levels of thrombocytes or leukocytes below the proposed reference interval will be a rest group which is indefinable.

Conclusion

Reference intervals are proposed for calves aged between 10 and 28 days of age. The proposed reference interval for thrombocytes is $300*10^9$/L - $1250*10^9$/L and $5.0*10^9$/L - $18.0*10^9$/L for leukocytes.

Conflict of interest statement

The author declares no conflicts of interest.

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Tables

Table 1
Calculated and proposed reference intervals for calves aged between 10-28 days. For method one calves were excluded on biological grounds, for method two calves with values in the top and bottom 2.5% for either thrombocytes or leukocytes were excluded**.**

	Method 1		Method 2	
Descriptives	Thrombocytes ($*10^9$/L)	Leukocytes ($*10^9$/L)	Thrombocytes ($*10^9$/L)	Leukocytes ($*10^9$/L)
Mean	754.4	9.44	775.7	9.47
Median	783.1	9.30	775.5	9.60
Standard deviation	1.4	1.43	241.7	1.39
Minimum	132.0	2.70	132.0	2.70
Maximum	1663.0	37.10	1322.0	18.70
Calculated reference interval	367.7 – 1547.7	4.64 – 19.20	302.0 – 1249.4	4.97 – 18.04
Proposed reference interval			300 – 1250	5.0 – 18.0

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Figure legends

Fig 1. Mean thrombocyte count in relation to age. Continues lines at 100 and 800 represent the reference interval for adult cattle (Knowles et al., 2000; Mohri et al., 2007; George et al., 2010).

Fig 2. Mean leukocyte count in relation to age. Continues lines at 4.0 and 12.0 represent the reference interval for adult cattle (Knowles et al., 2000; Mohri et al., 2007; George et al., 2010).

Fig 3. A = cases in the questionnaire (n=72), B = excluded calves in method one (n=68), C = included calves in method one (n=286) and D = included calves in method two (n=324). Calves aged from 10-28 days of age were used. For method one calves were excluded on biological ground, for method two calves with values in the top and bottom 2.5% for either thrombocytes or leukocytes were excluded**.** The figure shows the means and boxes of the two methods that have been used for defining the reference interval for thrombocytes. Lines at 100 and 800 represent the reference interval for adult cattle (Knowles et al., 2000; Mohri et al., 2007; George et al., 2010). Also the mean thrombocyte count of the cases is shown; the mean represents the value $<72*10^9$/L, because of the automatic which cannot detect values lower than $72*10^9$/L.

Fig 4. A = cases in the questionnaire (n=72), B = excluded calves in method one (n=68), C = included calves in method one (n=286) and D = included calves in method two (n=324). Calves aged from 10-28 days of age were used. For method one calves were excluded on biological grounds, for method two calves with values in the top and bottom 2.5% for either thrombocytes or leukocytes were excluded**.** The figure shows the means and boxes of the two methods that have been used for defining the reference interval for leukocytes. Lines at 4.00 and 12.00 represent the reference interval for adult cattle (Knowles et al., 2000; Mohri et al., 2007; George et al., 2010). Also the mean leukocyte count of the cases is shown.

Figures

Figure 1

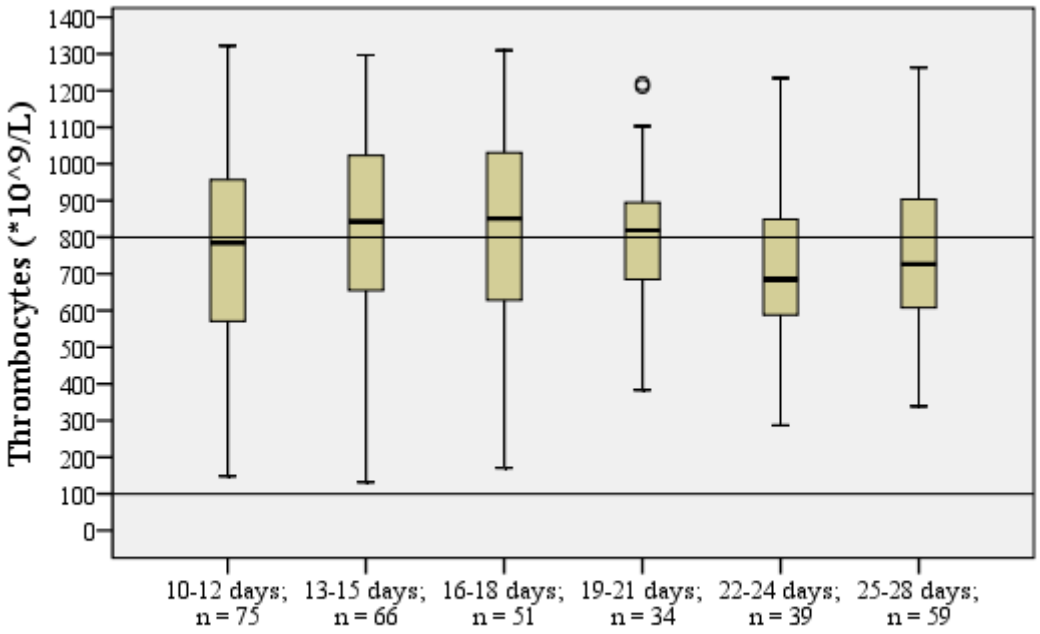


Figure 2

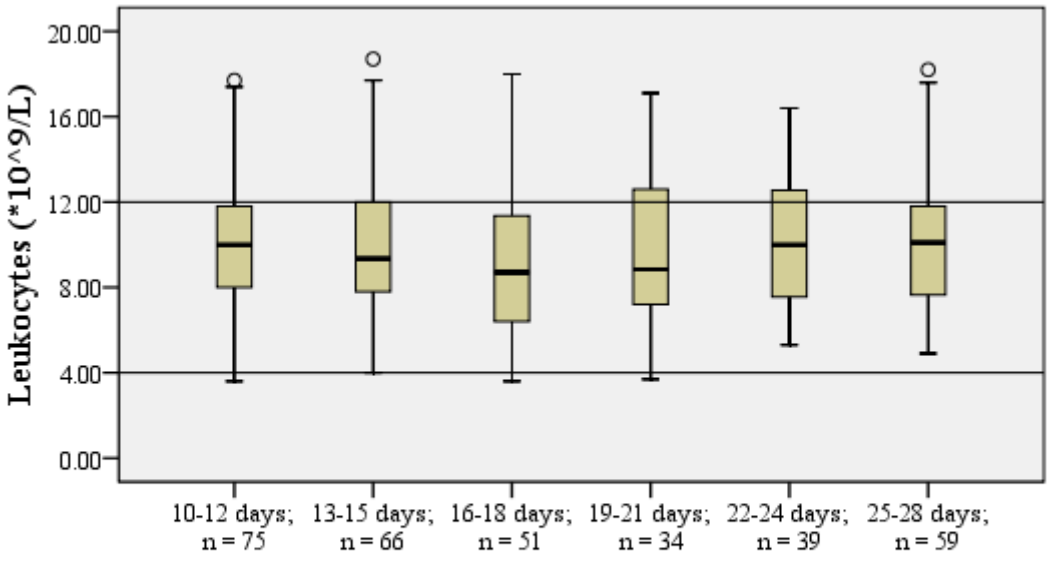
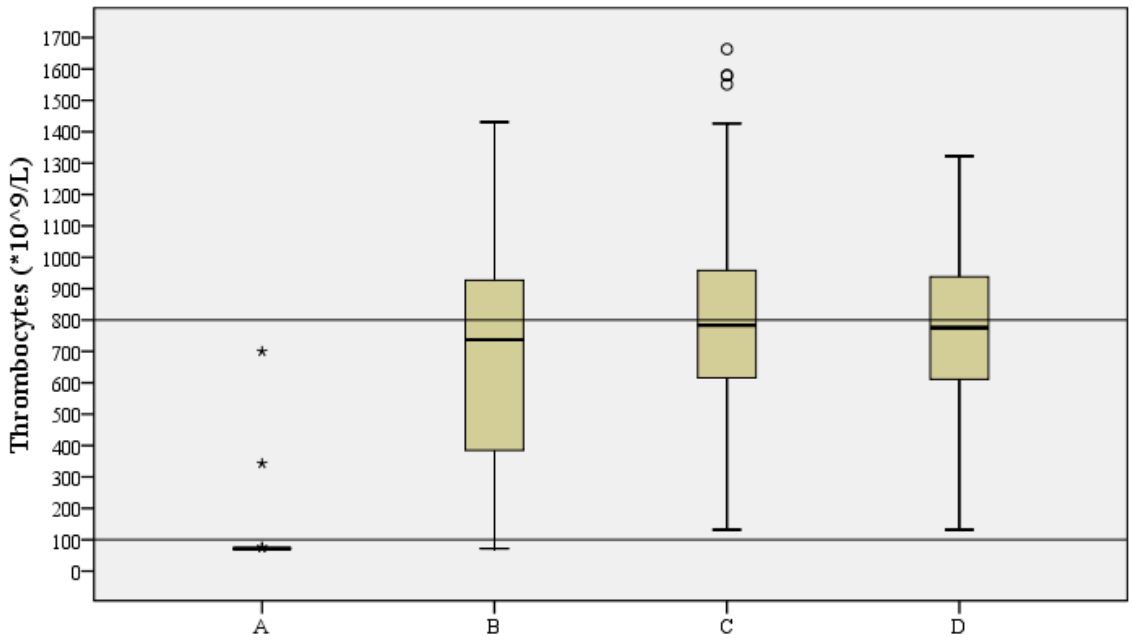


Figure 3


Thrombocytes (*10^9/L)
1700
1600
1500
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1300
1200
1100
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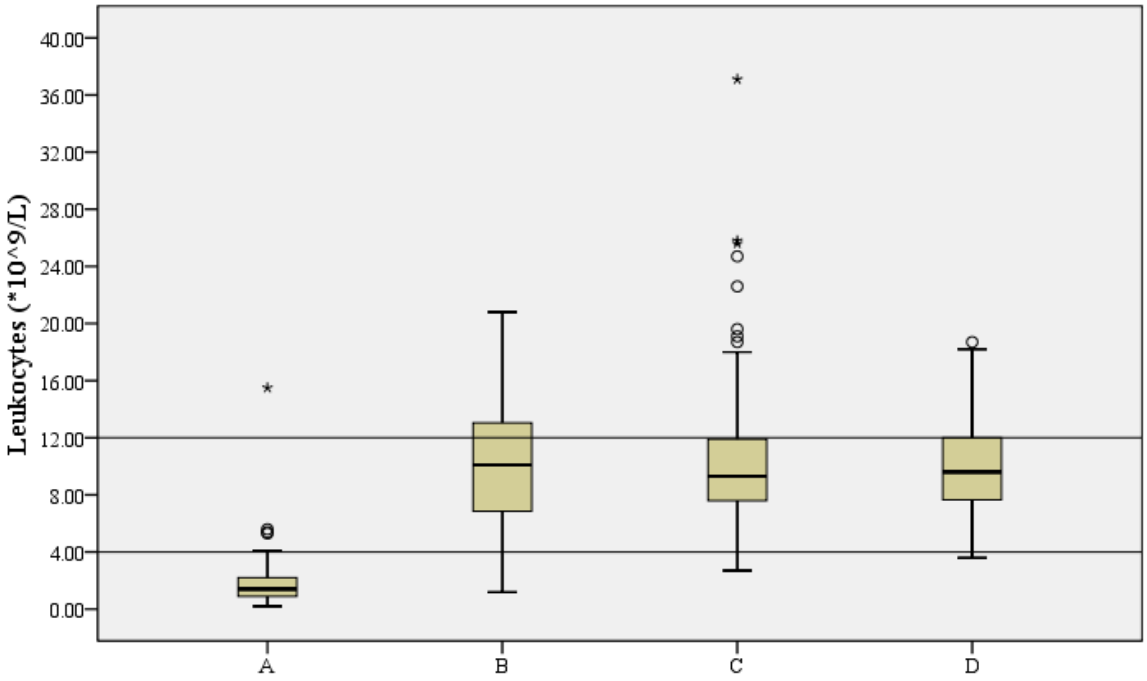
500
400
300
200
100
0
A
B
C
D

Figure 4


Leukocytes (*10^9/L)
40.00
36.00
32.00
28.00
24.00
20.00
16.00
12.00
8.00
4.00
0.00
A
B
C
D