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Detection of Endotoxins and Bacterial Agents in Collected Blood Bags and their Recipients

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Abstract: Bacterial contamination of blood and its cellular components remains an unresolved problem in transfusion medicine, and is considered to be the most common microbiological cause of transfusion associated morbidity and mortality. This is because contaminated units may contain large numbers of virulent bacteria as well as, endotoxins that are considered to be fatal to the recipients. Endotoxins are high-molecular weight complexes of lipopolysaccharides that constitute the major cell wall component in all Gram-negative bacterial families. These molecules have been intensively investigated because of the increasing appreciation of their potentially pathogenic role in a wide variety of human disease states. The present study aimed to detect endotoxins and bacterial agents in collected blood bags and their transmission to the recipients of these blood bags. The study involved 100 randomly selected blood bags and their recipients. They were all examined by Limulus Amebocyte Lysate (LAL) assay using gel clot method for detection of endotoxins and by blood culture for the detection of bacterial agents. Endotoxins were detected in five blood bags (5%) using LAL assay (gel clot method), while bacterial contamination was found in only one blood bag . The bag that gave positive blood culture yielded Staphylococcus aureus, which was mostly a skin associated organism and was considered as a contaminant related to the procedure during donor venipuncture. None of the 100 studied recipients of these blood bags revealed positive blood culture. It was concluded from this study that LAL assay is a rapid, easy to perform, and a highly sensitive test that can detect as little as 0.03 endotoxin units per ml using the gel-clot method. In addition not all endotoxins or bacterial agents could be transmitted to the recipients of blood bags, this depends on their volume and whether the recipient is on antibiotic therapy or not.

INTRODUCTION

Blood transfusion remains a common practice in the critical care, and in surgical settings. Transfusion carries significant risks, including risks for transmission of infectious agents, and several immune-modulator agents which are known to be related to cancer recurrences, mortality and post-operative infections.(1)

Bacterial contamination of blood and its cellular components is still an unresolved problem in transfusion medicine, and is considered to be the most common

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microbiological cause of transfusion associated morbidity and mortality. This is because contaminated units may contain large numbers of virulent bacteria as well as, endotoxins that are considered to be fatal to the recipients.[2]

Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria. They are invariably associated with Gram-negative bacteria whether the organisms are pathogenic or not. Endotoxin is a LPS composed of a variably antigenic series of sugars attached to a lipid-A moiety. The polysaccharide portion of the molecule consists of an O-specific chain and a core oligosaccharide.[3] These molecules have been intensively investigated because of the increasing appreciation of their potentially pathogenic role in a wide variety of human disease states.[4]

Endotoxin induces multiple biological effects in vivo, for example, fever, leukocytosis, hypoferremia, platelet aggregation, thrombocytopenia, and coagulopathies These effects can be attributed to activation of various endogenous pathways or cascade mechanisms. For example, LPS triggers the complement, coagulation, fibrinolytic, and kinin pathways to release vasoactive peptides and also the release of an array of cytokine mediators from macrophages and monocytes. The release of these mediators in turn triggers the characteristic biological effects.[4, 5]

There are more than 20 assays for the detection of endotoxins [4] of which three have been used for the detection of endotoxin in clinical specimens: The rabbit pyrogen assay, the Limulus Amebocyte Lysate (LAL) bioassay, and immunoassays. The method of choice would appear to be the LAL assay. The advantages of this assay are increased sensitivity, potential for quantification,

reactivity with the biologically active component lipid A, and relative convenience of operation.[6]

Transfusion transmitted bacterial contamination has been identified as the most common and severe infectious complication associated with transfusion. Approximately 57% of all transfusion transmitted infections and 16% of transfusion-related deaths have been associated with bacterial contamination. It has been estimated that 1 in 38,500 units of red cells, 1 in 3,300 units of random donor platelets, and 1 in 2,000 units of apheresis platelets are contaminated with bacteria.[7] In the U.S, bacterial contamination is considered the second most common cause of death from transfusion with mortality rates ranging from 1:20,000 to 1:85,000 donor exposures. Estimates of severe morbidity and mortality range from 100 to 150 transfused individuals per year.[8] In Egypt bacterial contamination affects up to 1-2% of the patients receiving platelets concentrates and up to 0.4% of those receiving RBCs.[9]

Bacterial contamination of blood components can occur under the following conditions: [10]

1. Collection of an asymptomatic donor who has an occult bacteremia at the time of donation, especially with Gram negative rods.
2. During the collection process with skin flora.
3. During the processing or storage of the blood component or use of the blood beyond its expiration date.

In cases of RBC contamination, several of the contaminated units appeared hemolyzed or had an unusual dark color. Symptoms started after as little as 20 ml to 50 ml of blood were infused and recipients developed septic shock due to an increased endotoxin level. [11] In the past few decades,

medical literature has shown many case studies of apparent sepsis predominantly due to Gram-positive bacteria from normal skin flora. [12]

The clinical severity of a transfusion-associated septic reaction can vary considerably, depending on: [8]

1. The species of bacteria present in the blood product unit, with Gram-negative organisms tending to cause more severe reactions, due to the presence of endotoxins often elaborated by such organisms.
2. The total number of bacteria infused or present in the cellular blood product unit infused to a recipient
3. The rate of propagation of the bacteria present.
4. Recipient characteristics, such as underlying disease, leukocyte count, the status of the immune system, and whether the recipient is receiving concomitant antibiotic therapy.

Aim of the work:

To study the presence of endotoxins and bacterial agents in collected blood bags and their transmission to the recipients of these blood bags at the Medical Research Institute.

Material & Methods:

The present study was conducted during 11 months period from April 2007 to February 2008 at the Medical Research Institute.

I. Sampling:

The study involved the collection of blood samples aseptically from:-

A-Blood bags

This was carried out on one hundred randomly selected blood bags. From each selected bag 10 ml of blood were aseptically withdrawn, after cutting the connection tube (the part attached to the blood bag) by sterile scissors, then connection tube was sealed.

b- Recipients of blood bags.

About 10 ml of venous blood were aseptically withdrawn from each recipient of the 100 studied blood bags with or without detectable endotoxins and from a phlebotomy site other than that used for transfusion. The studied recipients were admitted to the Medical Research Institute hospital, and had no signs or symptoms of infection before the transfusion process.

II- Data Collection: An information sheet was fulfilled for each blood bag; and a complete questionnaire sheet was filled for every recipient of a blood bag, they included all the relevant information (serial number, date of donation, brand name, age, sex, cause of transfusion…etc)

Each collected blood sample (from blood bags and their recipients) was divided into two portions; one of which was used for cultural procedures and the other was used for the detection of endotoxins.

A- Cultural procedures:

About 8ml of blood were aseptically inoculated into a biphasic blood culture bottle. Blood culture bottles were aerobically incubated at 37^{o}C and daily examined for evidence of bacterial growth (Turbidity and/or bacterial colonies on the slope) up to 7 days.[13, 14] Subculture was done on each of blood and MacConkey's agar plates from the blood culture bottle with evidence of bacterial growth or blindly after 48 hours of incubation. [15, 16] All plates were then aerobically incubated over night at 37^{o}C. Blood culture bottles were considered negative and discarded after 7 days.

Identification of bacterial isolates:

After over night incubation, isolated colonies growing on blood and MacConkey's agar plates were identified morphologically by microscopic examination using Gram staining and biochemical tests according to the standard methods described by Forbes *et al*.[17]

B-Detection of endotoxin by LAL assay:

About 2 ml of blood sample were aseptically transferred to a sterile empty non-pyrogenic labeled blood collection tube to be subjected to LAL assay; which was performed using the gel clot method and following the manufacturer's instructions.

Principle of LAL assay:

The test is used for detection of endotoxins in a given sample by LAL; depending on the fact that bacterial endotoxins (LPS) cause the *Limulus* blood to clot in a cascade of enzyme activation.

Treatment of blood samples:

1. Blood samples were centrifuged at 500 rounds per minute for 15 minutes to separate plasma fraction.
2. Separated plasma fractions were inoculated in non pyrogenic tubes using sterile disposable syringes.
3. Separated plasma fractions were diluted with LAL reagent water (LRW) 1:10.
4. The diluted samples were incubated in non circulating water bath at 78°C for 15 minutes.

Procedure:

1) About 0.2 ml of the test sample or control were directly added to the single test vial (STV). Each tube was
2) vortex-mixed for 1 to 2 seconds to ensure thorough mixing.
3) The reaction tubes were incubated at 37°C for 60 ± 2 minutes. The STVs were not disturbed during the incubation period.
4) The reaction tubes were removed and read one at a time. The tube was not bumped against the side of the rack. Each tube was inverted in one smooth motion; without pausing half way unless it was obvious that the gel has not formed.

Interpretation:

If a gel formed and remained intact in the bottom of the tube after inversion of 180°, the test was considered positive i.e.

the concentration of endotoxin in the tube is greater than or equal to 0.03 endotoxin units /ml (the sensitivity of the STV). Any other state of the mixture constituted a negative test and indicated an endotoxin concentration less than the STV sensitivity.

Results: The results of this study can be summarized as follows:

- Table 1 reveals that 5 blood bags were positive for endotoxins by LAL assay (5%) while only one recipient was positive for endotoxin after blood transfusion (1%). This case showed signs and symptoms of pyrogenic reactions following blood transfusion and suffered from multiple organ failure and died.
- Table 2 displays that almost all cultures of blood bags were negative (99%) and only one (1%) was positive. The only blood bag that showed positive blood culture yielded *Staphylococcus aureus*. None of the 100 studied recipients of these bags revealed positive blood culture (Zero%).
- It is clear from table 3 that most of positive blood bags for endotoxins by LAL assay (60%) have been stored for the duration of more than 7 days, while 40% have been stored for less than 7 days.
- It is evident from table 4 that the most commonly associated clinical condition among male recipients was liver cirrhosis which accounted for 30.6%, while among females the most commonly associated clinical condition was breast carcinoma which accounted for 42.1%.

Discussion:

Bacterial contamination of blood products is a persistent but over-looked problem in transfusion medicine, despite that the episodes of transfusion-associated sepsis may lead to fatal outcomes or other fatal

sequelae. [18] Knowledge of the prevalence of bacterial contamination of blood and its components for transfusion and the causes of contamination in different parts in the world are important for planning of preventive measures at blood transfusion centers and reduction of transfusion-transmitted bacterial infections.[19]

In the present study each collected blood sample (from the 100 studied whole blood bags and their recipients) has been subjected to traditional manual broth culturing technique for detection of bacterial agents. The only blood bag that gave positive blood culture yielded *Staphylococcus aureus*, and it was not transmitted to its recipient which revealed negative blood culture. This might be due to the fact that the recipient of this blood bag was on antibiotic therapy prior to transfusion. The isolate obtained in this study was mostly a skin associated organism and was considered as a contaminant related to the procedure during donor venipuncture.

Higher percentages of bacterial contamination in whole blood samples were reported by Adjei *et al*., (2009) in Ghana and Sagui *et al*., (1998) in Japan (9%and 6.3%) respectively which may reflect the situation in these transfusion centers. This could be due to donor bacteremia, inappropriate work- related behaviors practiced in these transfusion centers, poor storage conditions, and/or inadequate disinfection of the venipuncture site of blood donors.[19, 20]

On the other hand Kuehnert et al., (2001), Perez et al., (2001) and Brecher et al., (2002) reported lower rates of bacterial contamination of whole blood samples (0.1%, 0.2%, 0.15%) respectively, using the same cultural technique. [21-23] The lower rates of bacterial contamination in these studies could be attributed to the strict aseptic techniques used at the time of blood collection from donors.

In accordance with the results of this study, many studies reported that Gram-positive cocci were the most commonly isolated bacterial agents from blood and its products. [24-26] Fusa *et al*., (1998) recorded that the most commonly isolated microorganisms from blood were coagulase positive staphylococci (66.7%).[24]

In a French Hemovigilance study that was conducted in 1999, it was reported that 58% of the bacteria isolated from RBCs were Gram-positive cocci primarily *Staphylococcus* spp. and *Streptococcus* spp, while Gram-negative bacilli were identified in 32% of the cases.[25]

Also Okrah *et al.,* (2009), studied the bacterial agents responsible for the blood contamination at the Tamale Teaching Hospital in Ghana. They recorded that 71.42% were Gram positive cocci making them the commonest contaminants, of which 21.42% were *Staphylococcus aureus*. [26]

It is important to note that the problem of bacterial contamination is most challenging with platelets. The rate of infectious problems reported with platelets exceeds that with RBCs by 3:1 ratio.[27] This was documented by Morel *et al*., (2003) who demonstrated a high rate of bacterial contamination with *Bacillus spp.* and *Staphylococcus epidermidis* in platelet concentrates (41%). This is due to these organisms' survival and readily multiplication at temperatures of 20 º to 24 ºC; which are the storage temperatures of platelets.[28]

In the present study whole blood samples were cultured to detect bacterial agents. This might be the cause of the lower rate of bacterial contamination reported in this study than those in previous studies using platelet samples.

In the present study all the 100 studied blood bags were of the same Brand.

Plastic material for these blood bags has been specially formulated to meet required standards for biological safety. No problems such as cracking or breaking of bags have been encountered during centrifuge cycle or storage. Blood Bags are manufactured to the highest quality standards for optimum blood management including collection, separation, preservation and transfusion. (29)

Also collected blood may be contaminated endogenously as a result of asymptomatic bacteremia of the donor. (25) In the present study a careful questionnaire was fulfilled for all blood donors to exclude bacterial infections and high risk factors.

Definitive diagnosis of Gram-negative infections is usually made by identifying the organisms isolated from blood cultures, but often the results are not available for several days. (30)

The detection of endotoxins in the blood of patients with Gram-negative sepsis by means of an in vitro pyrogen test that depends upon the ability of endotoxin to produce gelation with an extract of blood cells (amebocytes) from horseshoe crab showed that there was a good correlation between the results of the test and the presence of Gram-negative infection. (30)

Finding appreciable amounts of endotoxins in the platelets of patients with Gram negative sepsis using LAL assay has been reported. (30)

In the present study each collected blood sample (from the 100 studied blood bags and their recipients) has been subjected to LAL assay (gel clot method) with sensitivity of 0.03 endotoxin unit per ml for detection of endotoxins. Five blood bags were positive for endotoxins (5%).

Because bacterial strains often proliferate in blood and its products during storage, some consideration has been given to shortening storage times (at least 2 weeks at 1º C to 6º C) in the hope of

reducing transfusion- associated sepsis.[19]

In the present study most of the blood bags with positive endotoxins (60%) were stored for more than 7 days. In concordance with the present study, Adjei *et al*., (2009) reported that the whole blood samples stored up to 1 week at 4°C recorded the highest levels of bacterial contamination (17.3%). [19]

It is known that the age of transfused blood is a risk factor for the development of multiple organ failure in surgical patients. However, the character of hemorrheological changes in stored blood as well as the time when they appear remains disputable. Berezina *et al* (2002) reported that serious hemorrheological disorders, including the decrease in RBC deformability secondary to shape abnormalities, acidosis, and the decrease of blood clotting, start already at the second week of storage and progress up to the end of the storage period. Transfusion of packed RBC older than 7 days may contribute to hemorrheological disorders in critically ill patients. [31] It is also important to note that the majority of septic transfusion reactions associated with contaminated RBCs usually occur with units that have been stored for more than 21 days. [8] The present study showed that the one case with positive endotoxin after blood transfusion has received blood which was stored for the duration of more than 7 days.

A septic reaction occurring during or following the transfusion of cellular blood components was one of the earliest recognized complications of blood transfusions. The presence of bacteria in blood products has been a problem for many decades and currently it is probably the most common microbiological cause of transfusion-associated morbidity and mortality. However, the transfusion to a recipient of a contaminated blood product

may not necessarily be associated with clinically evident morbidity. This is because the majority of contaminated blood product units contain only few bacteria. In other instances, contaminated units may contain large numbers of virulent bacteria as well as endotoxins, and their transfusion may be associated with significant morbidity and even be lethal to the recipient. (32)

In the present study, out of the 5 recipients of the blood bags that were positive for endotoxins, only one recipient was positive for endotoxins by LAL assay after transfusion. This case showed signs of pyrogenic reaction (fever, chills, and hypotension) and suffered from multiple organ failure and then died. This might be due to the fact that endotoxins may be fatal only if transmitted in large volume and in this study the detection of endotoxins was done only qualitatively and not quantitatively.

On the other hand, the recipient of the one blood bag that was positive for blood culture did not show any clinical signs of sepsis. This is similar to what was reported by Sagui *et al*., (1998) in Japan, where none of the patients who received culture-positive transfusion blood showed any clinical signs or laboratory findings of bacteremia. (20)

This study revealed that the associated clinical condition of most of the female recipients was breast carcinoma (42.1%). This might be attributed to higher incidence of anemia among patients with breast cancer as many of these patients suffer from anemia as a consequence of the disease itself or its treatment On the other hand, the most commonly associated clinical condition among male recipients was liver cirrhosis (30.6%).Cirrhosis is a complication of many liver diseases that is characterized by abnormal structure and function of the liver. (33) In a study which was carried out at Thailand university

hospital on 72 patients with liver cirrhosis with their ferritin levels of 200 ng/ml or less reported that about 40% of cirrhotic patients were anemic (iron deficiency anemia) and received blood transfusion.[34]

Liver cirrhosis is associated with dysregulation of the coagulation system resulting in an increased bleeding tendency in cirrhotic patients. The treatment approach to offset these abnormalities may involve transfusion with several types of blood products especially fresh frozen plasma and platelets. This association between need for blood transfusion and liver cirrhosis may be related to the fact that patients with cirrhosis are often affected by coagulopathy and portal hypertension. Furthermore, bacterial infections significantly increase the risk of bleeding and hospital mortality rate for patients with cirrhosis. [35-37]

It was concluded from this study that:

1. A complete & thorough questionnaire should be fulfilled for all blood donors to exclude bacterial infections and high risk factors.
2. LAL assay is a rapid, easy to perform, and a highly sensitive test. It can detect as little as 0.03 endotoxin units per ml using the gel-clot method.
3. Not all endotoxins and bacterial agents could be transmitted to the recipients of blood bags, it depends on their volume and whether the recipient is on antibiotic therapy or not.
4. Selection of blood bags with the highest quality standards is essential for optimum blood management including collection, separation, preservation and transfusion.
5. Long duration of storage of blood bags contribute to their contamination. In this study most of positive blood bags by LAL assay for endotoxins (60%) have been stored for more than 7 days.

Table 1: Results of LAL assay for endotoxins detection of the 100 studied blood bags and their recipients.

Results of LAL assay	Positive		Negative		Total	
	No.	%.	No.	%.	No.	%.
Blood bags	5	5	95	95	100	100
Blood recipients	1	1	99	99	100	100

Table 2: Results of blood cultures of the 100 studied blood bags and their recipients.

Results of blood cultures	Positive		Negative		Total	
	No.	%	No.	%	No.	%
Blood bags	1	1	99	99	100	100
Blood recipients	0	0	100	100	100	100

Table 3: Distribution of the five blood bags with positive LAL assay according to their duration of storage.

Duration of storage of blood bags	No.	%
Less than 7 days	2	40
More than 7 days	3	60

Table 4: Distribution of the 100 studied blood recipients according to the associated clinical condition and their sex.

Associated clinical condition	Male		Female	
	No.	%	No.	%
Iron deficiency anemia	4	9.6	1	2.6
B-Thalassemia major	3	4.8	2	5.2
Sickle cell anemia	2	3.2	1	2.6
Acute myeloblastic leukemia	8	12.9	4	10.5
Liver cirrhosis	19	30.6	2	5.2
Non Hodgkin's lymphoma	3	4.8	1	2.6
Multiple myeloma	2	3.2	2	5.2
Non calcular cholecystitis	1	1.6	2	5.2
Obstructive jaundice	2	3.2	2	5.2
Chronic renal failure	13	20.9	3	27.7
Breast carcinoma	0	0	16	42.1
Cancer colon	5	8.02	2	5.2
Total	62		38	

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