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Full Length Research Paper

Identification of contaminating microorganisms during collection of umbilical cord blood for transplantation

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ABSTRACT

Umbilical cord blood is a source of hematopoietic and mesenchymal stem cells for cellular therapy. Procedures to purify and to preserve these cells are performed under strictly controlled sterile conditions, but some units may become contaminated during collection. Ninety-six units detected as contaminated during an initial screening by Bact/Alert®3D60 (of 2,391 units collected from 2003 to 2012) were subjected to microbiologic evaluation by biochemical tests or Vitek-2 system to elucidate the probable origin of contamination. The most frequently isolated bacteria were from normal gastrointestinal flora such as *Escherichia coli* (24.6%), *Enterococcus sp [faecium* (19.7%)*, faecalis* (4.9%) and *gallinarum* (4.9%)]; *Citrobacter freundii* (6.5%); *Klebsiella pneumonia* (4.9%); *Proteus mirabilis* (3.3) and *Enterobacter cloacae (1.6%). Candida albicans* (19.7%) was the one yeast found. Normal constituent of the human skin and environmental bacteria were isolated: *Klebsiella pneumoniae* (4.9%) and *Acinetobacter calcoaceticus* 1.6%). *Pantoea agglomerans* (3.3%), *Cronobacter sakazakii* (1.6%), *Leclercia adecarboxylata* (1.6%) and *Staphylococcus lentus* (1.6%). The most probable contaminations sources were produced by cross contamination with perineal/enteric, vaginal or environmental microorganisms after delivering. The appointment of a specific trained person dedicated exclusively to blood collection and paying special attention on umbilical cord sanitization before puncturing might significantly reduce contamination.

Keywords: Umbilical cord blood, stem cells, transplantation, cross contamination.

INTRODUCTION

Umbilical cord blood (UCB) is used as a source of hematopoietic and mesenchymal stem cells for clinical applications in cellular therapy and regenerative medicine (Li et al., 2014)In comparison to bone marrow stem cells, or stem cells mobilized from bone marrow to peripheral blood and harvested by apheresis; umbilical cord blood stem cells (UCBSC) are more accessible, due to once they have been purified, analyzed, conserved and Human Leukocyte Antigens (HLA)-typified, they are immediately available for transplantation in compatible patients.

UCB collection is performed by methods that do not

ABBREVIATIONS

UCB, Umbilical cord blood; UCBSC, umbilical cord blood stem cells; UCBU, umbilical cord blood units;

involve the interference during a vaginal delivery or a caesarean section, due to it is performed when the newborn was already separated from its mother for neonatal care. In order to ensure UCBSC functionality for clinical use, cells must include several parameters during their purification. Initial blood volume, concentration of collected volume to a conservation volume, amount of viable mononuclear and hematopoietic cells, and absence of transfusion transmitted infectious diseases and contaminating aerobic and anaerobic microorganisms. Then, typifying the "ABO" blood group and HLA molecules and preservation of UCBSC in liquid nitrogen. After freezing and thawing, clonogenic assays are performed to verify their proliferation and differentiation capabilities. Only umbilical cord blood units (UCBU) complying 100% of selection criteria, are provide as suitable for clinical use; those UCBU that do not comply any selection criteria are discarded or used for research. All the methodological processes for UCBU validation for clinical use have to be performed under controlled sterile conditions, inside laminar flow cabinets in clean rooms with strict quality controls to guarantee sterility and proper operating conditions.

Collected volume of umbilical cord blood and amount of mononuclear and stem cells are selection criteria known before performing the volume reduction. Nevertheless, the absence of transfusion transmitted infectious diseases and contaminating microorganisms are obtained after UCBU freezing and storing (International standards for cord blood collection, banking, and release for administration, 2013; Brunstein and Wagner, 2006).

Since its founding, the Umbilical Cord Blood Bank from the National Center of Blood Transfusion from Mexico has detected seropositive UCBU for infectious agents analyzed during the serologic screening (HIV, HCV, HBsAg, *T. pallidum*, *T. cruzi*, *T. gondii*, Rubella, Cytomegalovirus and Herpes simplex virus). Seropositive units have been immediately discarded because they do not represent a choice for clinical treatment. This information has been analyzed to obtain the frequency of infectious agents in UCBU in our population. Additionally, some UCBU has been detected as contaminated with microorganisms; this screening only reports the presence of aerobic, anaerobic or both kinds of microorganisms (Riedel et al., 2009). Those contaminated UCBU have been also immediately discarded for clinical use, but labeled as "contaminated units" and stored in separated containers in liquid nitrogen. In this work, we identified the kind (bacteria or fungus) of contaminating microorganisms of UCBU; with the aim to elucidate the possible source of contamination, and in turn, to issue corrective actions and recommendations to prevent the contamination of UCBU during collection.

MATERIALS AND METHODS

Umbilical cord blood collection methodology

UCB collection was performed when the newborn and mother wellbeing had been verified and when placenta was still in uterus with the intention of harnessing contractions to drive blood through the umbilical cord. About 30 cm from the umbilicus a double clamping was performed in the umbilical cord where it was cut, a fragment was sanitized with povidone-iodine where a vein was punctured with one of two needles integrated in a cryobag (Grifols) with anticoagulant (CPD), maintaining a closed sterile system. The cryobag was filled by gravity, allowing the free blood flow through the bag to obtain the most possible amount of placental blood. The additional needle was used to obtain more blood when the first chosen vein collapsed, thereby preserving the close sterile system. UCBU were processed and analyzed to verify their suitability for clinical applications.

Volume reduction and sampling of contaminated umbilical cord blood units

The reduction from collected to conservation volume was performed in Sepax® systems. This system concentrated total nucleated cells (buffy-coat) in a cryobag of 20 mL, which had been stored in liquid nitrogen since its conservation until performing this work. The cryobag consists of two independent chambers, which can be detached separately; it also has three integrally attached segments used to facilitate any testing requirements for quality control in validated units before transplantation. The entire unit was overwrapped for added protection against contamination during storage. Ninety-seven UCBU detected as contaminated (of 2,391 UCBU collected from 2003 to 2012) with aerobic, anaerobic or both kinds of microorganisms during the initial screening by inoculating 5 mL of plasma in aerobic [(FA) bioMérieux] and anaerobic [(FN) bioMérieux] blood cultures and incubated in Bact/Alert® 3D60 system were used for this work. Sampling to identify the contaminating microorganisms was performed by using the fragments connected to the cryobag. The use of "not suitable for clinical applications UCBU" for research was authorized in the "informed consent" of the donors.

Isolation of contaminating microorganisms

Fragments obtained from the cryobag were sanitized with ethanol 70% and povidone-iodine, handled with sterile

material (scissors, forceps, syringes, etc), inside laminar flow cabinets. One sterile tube containing 5 ml of tryptic soy broth (TSB) (BD Bioxon, Mexico) and one sterile tube containing 5 mL of chopped meat carbohydrate broth (CMCB) (BD Diagnostics systems) were inoculated with 0.5 mL of UCB and incubated during 2 days at 37°C; tubes with TSB were incubated under aerobic conditions and tubes with CMCB under anaerobic conditions. Then tubes with visual turbidity were considered as positive growing; those with negative growing were incubated until 5 days more under the same incubation conditions. After 7 days of incubation, tubes with clear liquid mediums were considered as negative growing. Positive tubes were sampled to perform Gram staining (Sigma Aldrich) and to inoculate 1.0 mL of broth per culture media plate in order to get pure subcultures. Those positive under aerobic conditions were inoculated on plates of Blood Agar (BA), Sabouraud Dextrose Agar (SDA), Chocolate Agar (ChA) and MacConkey Agar (MCA) (BD Bioxon, Mexico). Those positive under anaerobic conditions were inoculated on plates of Blood Agar (BA) and Chocolate Agar (ChA) (BD Bioxon, Mexico). In order to detect colonies of microorganisms, plates were incubated under aerobic or anaerobic conditions respectively at 37°C, and analyzed every 24 hours up to 15 days,

Identification of contaminating microorganisms

The specie of Gram negative bacteria growing up in large colonies, big enough to perform all the tests, was identified by commonly used biochemical tests due to our Department of Microbiology has all this methods very well standardized for routine identification of enterobacteriaceae species. Bacteria growing up in small colonies and yeasts were identified by the VITEK-2 system in order to get highly dependable results.

The specie of Gram negative bacteria was identified from pure subcultures by commonly used biochemical tests (IMViC, [Indole test, Methyl red test, Voges-Proskauer test and Citrate test]; Sulphate reduction test; Urease test; PDA, Phenylalanine Deaminase test; LIA, Triple Sugar Iron Agar; TSI, Lysine Iron Agar; Motility and Ornithine descarboxylase. The specie of bacteria growing up in small colonies and the specie of yeasts were identified based on standard microbiological methods and additionally, each isolate was identified by the VITEK-2 system (bioMerieux) by using the GP-ID cards for Gram positive bacteria, the GN-ID cards for Gram negative bacteria and the YST-ID cards for yeasts; and following manufacturer instructions. Briefly, colonies from pure subcultures were suspended in 3.0 mL of sterile (aqueous 0.45% NaCl, pH 4.5) and turbidity was adjusted until achieve an equivalence to that of a McFarland between 0.5 – 0.63 to Gram positive or Gram negative bacteria, and between 1.8 – 2.20 to yeasts as measured by the DensiChek™ (bioMerieux) turbidity meter. The VITEK 2 system filled, sealed, and incubated every single test card, which were held at 35.5 ± 1 °C during 18 hours, with optical readings taken every 15 minutes. Based on those readings, an identification profile was established and interpreted according to a specific algorithm. Profiles were compared to the database, generating an identification of each microorganism. Identifications were classified as “excellent” if they were between 96 to 99% of probability, “very good” (93 to 95%), “good” (89 to 92%) and as “acceptable” (85 to 88%).

RESULTS

Features of Contaminated Umbilical Cord Blood Units

Ninety-seven CUCBU detected with positive growing for aerobic (30, 30.9%), anaerobic (25, 25.8%) or both kinds of microorganisms (42, 43.3%) during the initial screening by using Bact/Alert® 3D60 system (of 2,391 UCBU collected from 2003 to 2012) were used for this work. CUCBU were collected by vaginal birth or caesarean section as described in table 1 below. Various gynecology obstetricians and nurses from multifarious hospitals collaborated in UCB collection during that period; hence, we could not find any correlation between the number of CUCBU and a specific hospital or UCB collector.

Only 59 of 97 CUCBU presented turbidity in enrichment broths after 2 until 7 days of incubation. Twenty-one CUCBU were positive under aerobic conditions in TSB; Eight CUCBU were positive under anaerobic conditions in CMCB; and thirty CUCBU were positive under both aerobic and anaerobic conditions. All CUCBU with turbidity were sampled to perform Gram staining and to inoculate culture media plates in order to isolate pure colonies of the contaminating microorganisms as described in methods. Colonies were classified as large Gram negative, small Gram positive or small Gram negative, and Yeast colonies for analysis. Two CUCBU presented two kinds of colonies from the enrichment broth; units numbers 40 (Large Gram negative and small Gram-positive colonies) and unit number 49 (Large Gram negative and small Gram-negative colonies).

Microorganisms identified by biochemical tests

Twenty-seven large Gram-negative colonies isolated on culture media plates under aerobic and/or anaerobic conditions were analyzed by biochemical tests as mentioned in methods and described in table 2 below. Thirteen colonies isolated from CUCBU obtained by vaginal birth and two colonies isolated from CUCBU

Table 1. Features of Contaminated Umbilical Cord Blood Units.

Year of collection	2003	6	6.2 %
	2004	15	15.5 %
	2005	4	4.1 %
	2006	9	9.3 %
	2007	18	18.6 %
	2008	14	14.4 %
	2009	11	11.3 %
	2010	7	7.2 %
	2011	8	8.2 %
	2012	5	5.2 %
Kind of microorganism by initial screening by Bact/Alert® 3D60	Aerobic	30	30.9 %
	Anaerobic	25	25.8 %
	Aerobic/Anaerobic	42	43.3 %
Birth	Vaginal	64	66.0 %
	Caesarean	33	34.0 %

Ninety-seven CUCBU detected with positive growing for aerobic, anaerobic or both kinds of microorganisms during the initial screening by using Bact/Alert® 3D60 system were used for this work. UCBU were collected from 2003 to 2012, by vaginal or caesarean birth.

obtained by caesarean section, all of them coming from aerobic and anaerobic enrichment broths (facultative anaerobic microorganism), were biochemically characterized as IMViC (+,+,-,-), Sulfate reduction (negative), Urease (negative), Phenylalanine deaminase (negative), LIA (K/K), TSI (A/A), Motility (positive) and Ornithine descarboxylase (positive); therefore identified as *Escherichia coli.* Two colonies isolated from CUCBU obtained by only vaginal births, in both aerobic and anaerobic enrichment broths (facultative anaerobic microorganism), were biochemically characterized as IMViC (-,+,-,+), Sulfate reduction (positive), Urease (positive), Phenylalanine deaminase (positive), LIA (R/K), TSI (K/A), Motility (positive) and Ornithine descarboxylase (positive); thus identified as *Proteus mirabilis*. Two colonies isolated from CUCBU obtained by vaginal births and two colonies isolated from one CUCBU obtained by caesarean section, all of them coming from aerobic enrichment broths (aerobic microorganism), were biochemically characterized as IMViC (-,+,-,+), Sulfate reduction (positive), Urease (positive delayed), Phenylalanine Deaminase (negative), LIA (K/A), TSI (K/A), Motility (positive) and Ornithine (weak), hence identified as *Citrobacter freundii*. Three colonies isolated from CUCBU obtained only by vaginal births, in both aerobic and anaerobic enrichment broths (facultative anaerobic microorganism), were biochemically characterized as IMViC (-,-,+,+), Sulfate reduction (negative), Urease (positive), Phenylalanine Deaminase (negative), LIA (K/K), TSI (A/A), Motility (negative) and Ornithine (negative); therefore identified as *Klebsiella pneumoniae*. One colony isolated from one CUCBU obtained by vaginal birth and one colony isolated from one CUCBU obtained by caesarean section, coming from aerobic enrichment broths (aerobic microorganism), were biochemically characterized as IMViC (-,-,+,+), Sulfate reduction (negative), Urease (positive delayed), Phenylalanine Deaminase (negative), LIA (K/K), TSI (K/A), Motility (positive) and Ornithine (negative); thus identified as *Pantoea agglomerans*. One colony isolated from one CUCBU obtained by vaginal birth, coming from one anaerobic enrichment broth (anaerobic microorganism), was biochemically characterized as IMViC (-,-,+,+), Sulfate reduction (negative), Urease (positive delayed), Phenylalanine Deaminase (negative), LIA (K/K), TSI (A/A), Motility (positive) and Ornithine (positive), hence identified as *Enterobacter cloacae* (MacFaddin, 2000).

Microorganisms identified by assimilation profiles on Vitek-2 system

Twenty two small Gram negative or Gram positive colonies and twelve yeast colonies isolated on culture media plates under aerobic and/or anaerobic conditions were analyzed by assimilation profiles on Vitek-2 system as mentioned in methods and described in table 3 (Gram-positive and Gram-negative bacteria) and table 4 (yeasts). Probabilities of identification were always over 91% and the confidence levels were from “Good” to “Excellent”.

Enterococcus faecium was identified in seven colonies isolated from CUCBU obtained by vaginal births and five colonies isolated from CUCBU obtained by caesarean sections, coming from aerobic and anaerobic enrichment broths (facultative anaerobic microorganism). *Enterococcus faecalis* was identified in three colonies

Table 2. Biochemical analysis of large Gram-negative colonies.

Unit number	Initial screening by Bact/Alert® 3D60		Birth	Growing in enrichment broth		Gram Staining	Biochemical tests											Microorganism identified
	AE	ANA		TSB AE	CMCB ANA		I	MR	VP	Cit	H_2S	Ur	PDA	LIA	TSI	M	O	
2	-	+	V	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
4	+	+	V	+	+	-	-	+	-	+	+	+	+	R/A	K/A	+	+	*Proteus mirabilis*
8	-	+	V	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
9	+	-	V	+	-	-	-	+	-	+	+	+ D	-	K/A	K/A	+	W	*Citrobacter freundii*
14	+	+	V	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
26	+	+	V	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
36	+	+	V	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
39	+	+	V	+	+	-	-	+	-	+	+	+	+	R/A	K/A	+	+	*Proteus mirabilis*
40	+	+	V	+	+	-	-	-	+	+	-	+	-	K/K	A/A	-	-	*Klebsiella pneumoniae*
42	-	+	V	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
44	-	+	V	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
47	+	+	V	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
51	+	-	V	+	-	-	-	+	-	+	+	+ D	-	K/A	K/A	+	W	*Citrobacter freundii*
60	+	+	V	+	+	-	-	-	+	+	-	+	-	K/K	A/A	-	-	*Klebsiella pneumoniae*
65	+	-	V	+	+	-	-	-	+	+	-	+	-	K/K	A/A	-	-	*Klebsiella pneumoniae*
68	+	+	V	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
71	+	+	V	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
73	+	-	V	+	-	-	-	-	+	+	-	+ D	-	K/K	K/A	+	-	*Pantoea agglomerans*
77	+	-	V	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
79	-	+	V	-	+	-	-	-	+	+	-	+ D	-	K/K	A/A	+	+	*Enterobacter cloacae*
88	+	+	V	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
22	+	+	C	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
27	+	-	C	+	-	-	-	+	-	+	+	+ D	-	K/A	K/A	+	W	*Citrobacter freundii*
38	+	+	C	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
48	+	+	C	+	+	-	+	+	-	-	-	-	-	K/K	A/A	+	+	*Escherichia coli*
49	+	-	C	+	-	-	-	+	-	+	+	+ D	-	K/A	K/A	+	W	*Citrobacter freundii*
78	+	-	C	+	-	-	-	-	+	+	-	+ D	-	K/K	K/A	+	-	*Pantoea agglomerans*

Large Gram-negative colonies isolated on culture media plates under aerobic and/or anaerobic conditions were analyzed by biochemical tests as mentioned in methods. Abbreviations: + D = Positive delayed; A = Acid; AE = Aerobic; ANA = Anaerobic; C = Caesarean birth; Cit = Citrate; CMCB = Chopped Meat Broth; H2S = H2S production in SIM; I = Indol; K = Alkaline; M = Motility; MR = Methyl red; O = Ornithine descarboxylase; PDA = Phenylalanine Deaminase; TSB = Trypticase Soya Broth; UR= Urease; V = Vaginal birth; VP = Voges-Proskauer; W = Weak.

Table 3. Gram positive and Gram negative bacteria identified by assimilation profiles on Vitek-2 system.

Unit number	Initial screening by Bact/Alert® 3D60		Birth	Growing in enrichment broth		Gram Staining	Probability by VITEK-2 system	Confidence Level	Microorganism identified
	AE	ANA		TSB AE	CMCB ANA				
1	-	+	V	-	+	+	93%	VG	*Enterococcus faecalis*
21	+	-	V	+	+	+	98%	E	*Enterococcus faecium*
31	-	+	V	-	+	+	98%	E	*Enterococcus faecium*
35	+	+	V	+	+	+	97%	E	*Enterococcus gallinarum*
40	+	+	V	+	+	+	98%	E	*Enterococcus faecium*
53	+	+	V	+	+	+	93%	VG	*Enterococcus faecium*
74	-	+	V	-	+	+	99%	E	*Enterococcus gallinarum*
80	+	-	V	+	+	+	93%	VG	*Enterococcus faecalis*
84	-	+	V	-	+	+	97%	E	*Enterococcus faecium*
85	+	+	V	+	+	+	93%	VG	*Enterococcus faecium*
87	+	-	V	+	+	+	92%	G	*Enterococcus faecalis*
94	+	+	V	+	+	+	97%	E	*Enterococcus faecium*
41	+	-	V	+	-	-	91%	G	*Acinetobacter calcoaceticus*
15	+	+	C	+	+	+	98%	E	*Enterococcus gallinarum*
17	+	+	C	+	+	+	99%	E	*Enterococcus faecium*
32	+	+	C	+	+	+	93%	VG	*Enterococcus faecium*
45	+	+	C	+	+	+	97%	E	*Enterococcus faecium*
57	+	+	C	+	+	+	91%	G	*Staphylococcus lentus*
62	+	+	C	+	+	+	94%	VG	*Enterococcus faecium*
82	+	-	C	+	+	+	*93%*	VG	*Enterococcus faecium*
49	+	-	C	+	-	-	95 %	VG	*Leclercia adecarboxylata*
66	+	-	C	+	-	-	91%	G	*Cronobacter sakazakii*

Gram negative or Gram-positive small colonies isolated on culture media plates under aerobic and/or anaerobic conditions were analyzed by assimilation profiles on Vitek-2 system as described in methods. Abbreviations: AE = Aerobic; ANA = Anaerobic; C = Caesarean birth; CMCB = Chopped Meat Broth; E = Excellent; G = Good; TSB = Trypticase Soya Broth; V = Vaginal birth; VG = Very Good.

Table 4. Yeasts identified by assimilation profiles on Vitek-2 system.

Unit number	Initial screening by Bact/Alert® 3D60		Birth	Growing in enrichment broth		Probability by VITEK-2 system	Confidence Level	Microorganism identified
	AE	ANA		TSB AE	CMCB ANA			
10	+	+	V	+	-	99%	E	*Candida albicans*
16	+	+	V	+	-	99%	E	*Candida albicans*
33	+	+	V	+	-	96%	E	*Candida albicans*
43	+	-	V	+	-	92%	G	*Candida albicans*
86	+	-	V	+	-	97%	E	*Candida albicans*
93	+	-	V	+	-	98%	E	*Candida albicans*
97	+	-	V	+	-	97%	E	*Candida albicans*
12	+	-	C	+	-	95%	VG	*Candida albicans*
54	+	-	C	+	-	99%	E	*Candida albicans*
76	+	+	C	+	-	97%	E	*Candida albicans*
91	+	-	C	+	-	99%	E	*Candida albicans*
95	+	-	C	+	-	99%	E	*Candida albicans*

Yeast colonies isolated on culture media plates under aerobic and/or anaerobic conditions were analyzed by assimilation profiles on Vitek-2 system as described in methods. Abbreviations: AE = Aerobic; ANA = Anaerobic; C = Caesarean birth; CMCB = Chopped Meat Broth; E = Excellent; G = Good; TSB = Trypticase Soya Broth; V = Vaginal birth; VG = Very Good.

isolated from CUCBU obtained only by vaginal births, coming from aerobic and anaerobic enrichment broths (facultative anaerobic microorganism). *Enterococcus gallinarum* was identified in two colonies isolated from CUCBU obtained by vaginal births and one colony isolated from CUCBU obtained by caesarean section,

coming from aerobic and anaerobic enrichment broths (facultative anaerobic microorganism). *Acinetobacter calcoaceticus* was identified in one colony isolated from a CUCBU obtained by vaginal birth, coming from aerobic and anaerobic enrichment broths (facultative anaerobic microorganism). *Staphylococcus lentus* was identified in one colony isolated from a CUCBU obtained by caesarean section, coming from aerobic and anaerobic enrichment broths (facultative anaerobic microorganism). *Leclercia adecarboxylata* was identified in one colonies isolated from a CUCBU obtained by caesarean section, coming only from aerobic enrichment broths (aerobic microorganism). *Cronobacter sakazakii* was identified in one colonies isolated from a CUCBU obtained by caesarean section, coming only from aerobic enrichment broths (aerobic microorganism).

Candida albicans was the one yeast identified in all the colonies isolated from CUCBU obtained by vaginal or cesarean births, all of them coming from aerobic enrichment broths (aerobic microorganism) but from aerobic and/or anaerobic conditions (facultative aerobic microorganism) during the initial screening by Bact/Alert® 3D60.

DISCUSSION

In this study, we identified contaminating microorganisms of UCBU with the aim to detect probable contaminating sources and to issue recommendations to prevent their contamination during collection. Ninety-seven CUCBU detected with positive growing during the initial screening by Bact/Alert® 3D60 system were used for this work; nevertheless, only 59 presented turbidity in aerobic and/or anaerobic enrichment broths. In two enrichment broths, the presence of two kinds of microorganisms was found. Finally, we could identify 14 different microorganisms in 61 microbiological isolates.

Sampling of CUCBU was performed in the three segments attached to the cryobags in order to leave enough umbilical cord blood to accomplish other investigations about stem cells. We consider that the thirty-eight CUCBU with negative growing in enrichment broths might be due to microbial bioburden was not high enough to be homogeneously distributed between cryobag and fragments during separation done by a dielectric sealant. Additionally, owing to the CUCBU had been selected by the initial screening performed by sampling the plasma obtained during the concentration of the UCB to get the conservation volume; the bioburden in segments could be very low or absent in comparison to the bioburden in plasma. Some microorganisms present in CUCBU with low bioburden might be sensitive to freezing and thawing as demonstrated to salmonella and coliforms (Gunnarsdóttir et al., 2012), and to *Bacillus subtilis*, *Escherichia coli*, *Clostridium sporogenes*, and *Propionibacterium acnes* (Clark et al., 2014); therefore it could be why these microorganisms did not grow up in enrichment broths. Nevertheless, the number of contaminating microorganisms identified in CUCBU is noteworthy and enough to infer about possible contamination sources, because similar to other studies published previously (Clark et al., 2012; Zhu et al., 2013), our study reveal that microbial contamination of UCBU might be mainly during collection. Additionally our results revealed the presence of fourteen contaminating microorganisms acquired throughout ten years of UCB collection performed by diverse collectors and from various hospitals, showing the same contamination sources that have to be attended and eliminated.

The most frequently isolated contaminating microorganism was *Escherichia coli* (15 units, 24.5%). This enterobacterium as well as other microorganism identified such as *Candida albicans; Enterococcus sp [faecium, faecalis* and *gallinarum]; Citrobacter freundii; Klebsiella pneumonia; Proteus mirabilis* and *Enterobacter cloacae* are described as normal flora of the gastrointestinal tract in humans (Marathe et al., 2012). These bacteria were identified in CUCBU coming from 39 vaginal births (63.9%) and 16 caesarean sections (26.2%), all together represent 90.2% of the identified microorganisms, indicating that either vaginal or caesareans births the most frequent contamination source comes from cross contamination with perineal/enteric microbial flora. Other contaminations sources can also be inferred since *Candida albicans* has been also widely described as normal vaginal flora (Gondo et al., 2011). *Klebsiella pneumoniae* (Pinto-Almeida et al., 2012) and *Acinetobacter calcoaceticus* (Nonaka et al., 2014) are normal constituent of human skin. *Pantoea agglomerans* (Vaiman et al., 2013), *Cronobacter sakazakii* (Jung et al., 2013), *Leclercia adecarboxylata* (Keren et al., 2014) and *Staphylococcus lentus* (Adegoke, 1986) are ubiquitous environmental microorganisms (table 5); all of them may produce UCB contamination after birth due to a poor sanitization of the umbilical cord or the delivery rooms.

Under normal conditions the human fetus, the placenta and the umbilical cord are sterile; first, they are exposed to microorganisms of the vaginal flora when passing through the birth canal. The exposure to environmental microbes continues after birth when they are in contact with materials and people of the delivery team (Stumpf et al., 2008). Delivery rooms and procedures for attending births are designed to provide maximum reduction of exogenous microorganisms, which could contaminate the operative area and to origin postpartum infections in the mother and/or the newborn (Leth et al., 2009). Nevertheless, bowel movements are frequent and unavoidable incidents during vaginal deliveries, the medical team is used to it and they clean and sanitize immediately. Nevertheless, umbilical cord blood

Table 5. Probable contamination source of UCBU.

Microorganism identified	N° Vaginal birth (%)	N° Caesarea section (%)	Total (%)	Probable contamination source
Escherichia coli	*12 (19.7)*	*3 (4.9)*	15 (24.6)	Gastrointestinal tract.
Candida albicans	*7 (11.5)*	*5 (8.2)*	12 (19.7)	Vagina or gastrointestinal tract.
Enterococcus faecium	*7 (11.5)*	*5 (8.2)*	12 (19.7)	Gastrointestinal tract.
Citrobacter freundii	*2 (3.3)*	*2 (3.3)*	4 (6.5)	Gastrointestinal tract.
Enterococcus faecalis	*3 (4.9)*	*0 (0.0)*	3 (4.9)	Gastrointestinal tract.
Enterococcus gallinarum	*2 (3.3)*	*1 (1.6)*	3 (4.9)	Gastrointestinal tract.
Klebsiella pneumoniae	*3 (4.9)*	*0 (0.0)*	3 (4.9)	Human skin or gastrointestinal tract.
Pantoea agglomerans	*1 (1.6)*	*1 (1.6)*	2 (3.3)	Ubiquitous, environmental.
Proteus mirabilis	*2 (3.3)*	*0 (0.0)*	2 (3.3)	Gastrointestinal tract, environmental.
Acinetobacter calcoaceticus	*1 (1.6)*	*0 (0.0)*	1 (1.6)	Human skin, environmental.
Cronobacter sakazakii	*0 (0.0)*	*1 (1.6)*	1 (1.6)	Ubiquitous, environmental.
Enterobacter cloacae	*1 (1.6)*	*0 (0.0)*	1 (1.6)	Gastrointestinal tract.
Leclercia adecarboxylata	*0 (0.0)*	*1 (1.6)*	1 (1.6)	Ubiquitous, environmental.
Staphylococcus lentus	*0 (0.0)*	*1 (1.6)*	1 (1.6)	Animal skin, environmental
Total	*41 (67.3)*	*20 (32.7)*	61 (100)	

Fouthteen microorganisms were identified from 61 colonies isolated from 59 enrichment broths with positive growing from 97 UCBU identified as contaminated with aerobic or/and anaerobic microorganisms by the initial screening. The table shows the number and percentages of the probable contamination source based on the natural habitat of the microorganisms identified.

collection is a secondary event during deliveries, because as it is expected, obstetricians are mainly focused on the newborn and mother wellbeing and usually they do not carefully attend the directions for blood collection. Therefore, owing to our results indicate that the most probable contaminations sources are produced by cross contamination with perineal/enteric, vaginal or environmental microorganisms after delivering; we recommend to appoint and to capacitate a specific person of the medical team to be exclusively dedicated to umbilical cord blood collection. This person should at first allow the obstetricians work and do not interfere in the free flow of delivering, ensuring the newborn and mother wellbeing; then this person should pay special attention on umbilical cord sanitization before puncturing and to get the most possible volume of umbilical cord blood. Furthermore, different disinfectants, techniques, application times and procedures might be tested in each cord blood bank in order to establish their own best conditions to ensure avoiding cord blood contamination during collection.

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