Cytographic changes on BC-6800 Haematological Analyzer related to the presence of *Candida albicans* in peripheral blood. A new tool to suspect candidemia?

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ABSTRACT

Background We studied the quantitative and cytographic changes that the presence of *Candida albicans* (*C. albicans*) in peripheral blood (PB) samples causes on the Mindray BC-6800 Haematological Analyzer.

Methods A simulated in vitro candidemia was obtained by adding a different amount of *C. albicans* to discarded remnants of PB samples. Quantitative data and cytographic features were evaluated immediately as well as after 120 and 240 min of the yeast addition. A microscopic slides review was even performed at the same time.

Results After yeasts addition, an increase of total leucocytes, neutrophils and basophils have been observed, but these increases are not certainly descriptive of *C. albicans* presence.

Instead, extracellular blastospores cause a false increase in nucleated red blood cells (nRBCs), which appear as a new population in the specific counting channel for erytroblasts (NRBC channel). Regardless of the numbers, *C. albicans* form a pseudo-erythroblastic cluster in the NRBC channel whose resulting shape is so different than the 'normal' nRBC that it demands a microscopic review. Even cytographic changes related with the neutrophilic phagocytic activity have been observed on leucocyte's differential count citogram (DIFF) of the BC-6800.

Conclusions Our observations suggest that the results of the BC-6800, which are due to *C. albicans*' presence, might be useful to speculate earlier diagnosis of sepsis.

INTRODUCTION

Candida infections are a relevant and increasing problem in debilitated and immunosuppressed patients who can evolve from a superficial non-life-threatening disease to a clinical picture of severe sepsis with multiorgan-associated dissemination. This last condition is burdened by a high mortality rate especially when an adequate antifungal therapy is not started. So, it has been recommended that an empirical antifungal therapy be undertaken even before the results of blood culture are ready.[1–3] For this need, microscopic review of peripheral blood (PB) smears as well as the effects of yeast presence on automated cell counting might be useful.[4–7]

We have recently reported that the presence of *Candida parapsilosis* circulating in PB as well as the neutrophils (NE) containing phagocytised yeast blastospores cause morphological changes in cytograms of BC-6800 and that these cytographic features can be helpful in an earlier diagnosis of sepsis caused by this yeast.[8] Since *Candida albicans* is the most prevalent species that can cause yeast bloodstream infections (BSIs),[9 10] we evaluated if the cellular counting and cytographic anomalies of the BC-6800 Analyzer could be even useful in these cases just like as observed in cases of *C. parapsilosis* sepsis. For these purposes, we examined the quantitative and cytographic information that could be acquired by processing PB samples containing various amounts of *C. albicans* in the BC-6800 Analyzer.

MATERIALS AND METHODS

Study design

The interferences in the analytical results caused by yeast presence in PB were studied by simulating a sepsis in vitro.[7 11] For this purpose, various amounts of *C. albicans* were added to PB samples to simulate BSI, which in vivo are characterised by the presence of both extracellular and phagocytised yeasts.[1 2 8] Each blood sample was analysed in the BC-6800 in different times. Quantitative and cytographic changes in the BC-6800 Analyzer as well as morphological features from slide review have been evaluated. The study was approved by the local ethics committee and was performed according to the Declaration of Helsinki.

C. albicans suspension

Simulated candidemia was obtained by adding variable volumes of *C. albicans* suspension to each sample to obtain different final yeast concentration in each tube. For this purpose, a *Candida* from clinical material was typed as *C. albicans* by MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) system (Vitek; bioMérieux Italia, Florence, Italy) and subcultured on Sabouraud dextrose agar (SDA) (Vacutest Kima, Arzergrande, Italy). Isolated colonies were transferred on a brain–heart infusion (BHI) broth (Biolife, Milan, Italy) and stored in incubator at 37°C for 48 hours before use. Throughout the study, 5 different primary cultures of *C. albicans* and altogether 15 subcultures were used.

After 5 min of mixing by vortex, an aliquot of *C. albicans* suspensions was diluted in 5 mL of

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normal saline and adjusted by transmittance at 530 nm (Spectronic 20D; Milton Roy, Rochester, New York, USA) to 0.5 McFarland standard (Becton Dickinson, Sparks, Maryland, USA) corresponding approximately to 1×10^9 to 5×10^9 yeasts/L. The yeast concentration on BHI stock suspension was then calculated.

Preliminarily, the *C. albicans* vitality was evaluated regarding their aptitude to be phagocytised as well as their growth in blood culture bottle just as in clinical samples.

For the first purpose, a drop of *C. albicans* suspension was added to a drop of each blood sample used and the mixture was observed while fresh under cover slip at ×400 magnification to search cells that were engulfing or had engulfed yeasts (figure 1). An arbitrary cut-off equal to 10 engulfing cells was established. Later, for the evaluation of growth, a few amount from each of 15 new subcultures was added to PB samples in K_2EDTA tubes (final concentration ranging from 0.020×10^9 to 0.040×10^9 yeasts/L) and they were inoculated in the BACTEC Peds Plus F culture vials. Each of these was incubated on the BACTEC 9240F system (Becton Dickinson, Franklin Lakes, New Jersey, USA) until signal was positive or till the end of day 5. When positive signal was observed, bottles were unloaded from instrument. Gram's stain and cultures were performed according to standard microbiological protocol to exclude bacterial or fungal contamination standard microbiological protocol to exclude bacterial or fungal contamination.

Cytographic features on DIFF, BASO and nucleated red blood cell (nRBC) channels in the BC-6800 were evaluated.

Samples

This study was performed on 142 discarded remnants of PB samples collected for clinical purposes in K_2EDTA tubes. The analysis of the impact of the presence of *C. albicans* was carried out by adding variable amounts of yeast to samples as follows:

Group A: 51 samples without nRBC. In each sample, variable amounts of *C. albicans* were added to reach final concentration in PB that ranged from 0.20×10^9 to 4.0×10^9 yeasts/L. Quantitative and/or qualitative changes of haematological data were evaluated immediately (T0), at 120 min (T120) and at 240 min (T240) after yeast addition.

Group B: 21 samples in which nRBC were present initially (from 0.017×10^9 to 0.809×10^9/L). Even in these tubes, variable amounts of *C. albicans* were added to reach final concentrations ranging from 0.03×10^9 to 1.30×10^9 yeasts/L.

Group C: 70 samples without nRBC to determine the lower amount of added yeasts capable of causing cytographic changes as well as interferences in the cells blood count (CBC) and leucocytes differential count (LDC) in the BC-6800 Analyzer. Only T0 counting was performed in this group after addition of *C. albicans* to reach a final concentration ranging from 0.02×10^9 to 0.20×10^9 yeasts/L.

From CBC and LDC, we considered the sensible parameters for our purpose: total white blood cells (WBC) and platelets (PLT); absolute value of NE, basophils (BA) and nRBC. All T0 counts were performed within 2 hours of collection and samples from T0 to T240 were stored at room temperature.

Complete blood count and leucocyte differential count

CBC and LDC were performed with the BC-6800 Haematological Analyzer (Mindray, Shenzhen, China). In this analyzer, RBC and PLT are counted by using aperture impedance methodology. WBC count and LDC are obtained from three laser optical channels named DIFF, Basophils (BASO) and NRBC. LDC and nRBC are performed in the respective channels by using side and forward laser scatter analysis (SS and FS) as well as by fluorescence (FL). The resulting three-dimensional scattergrams can be rotated to allow a detailed observation of cell population clusters, their complexity, their size and their nucleic acids content on SS, FS and FL axes, respectively (figure 2).

Microscopic review

PB smears were performed and stained with May Grünwald-Giemsa (Merck, Darmstadt, Germany) with an automated slide maker SP-1000i (Sysmex, Kobe, Japan). Microscopic review has been made on light microscope (×1000 magnification) by experienced operators. All 142 selected samples have been observed before and after yeast addition in light microscopy up to 200 leucocytes according to internal procedure. In addition, in 25 out of 51 group A samples, blood smears were prepared and observed by two operators up to 200 NE even at T120 and T240 timing to evaluate the percentage of NE engulfing yeasts.

Statistical analysis

Significance of the differences of the various parameters obtained in paired samples measured with the BC-6800 were evaluated according to Steel-Dwass-Critchlow-Fligner test, with assessment of Hodges-Lehmann location shift for multiple

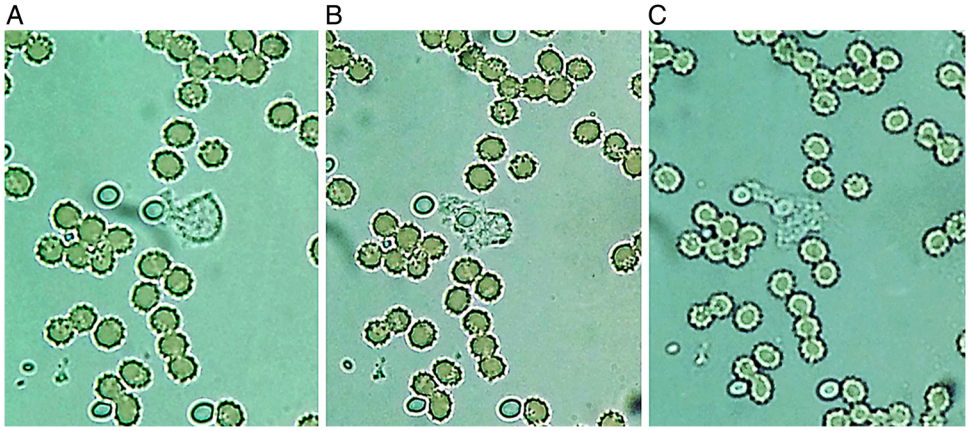

Figure 1 In vivo phagocytic activity. Mixture of peripheral blood and *Candida albicans* while fresh, ×400 magnification. (A) Phagocytic cell started the engulfment process after issuing a pseudopod. (B) Five minutes later, the first blastospore is completely inside the phagocytic cell. (C) Three minutes later, a new pseudopod is issued and the phagocytic activity continues to engulf the closer blastospore.

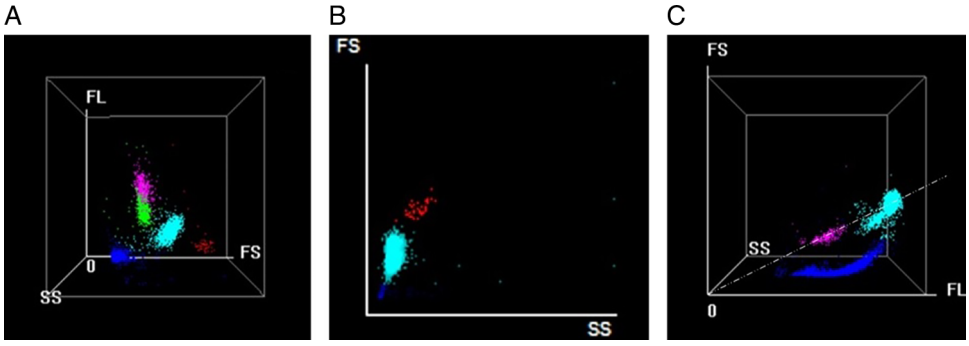

Figure 2 Normal sample: DIFF, BASO and NRBC channels of BC-6800. (A) DIFF cytogram is rotated 90° to the left. Four clusters are present: neutrophils (azure), eosinophils (red), monocytes (fuchsia) and lymphocytes (green). (B) BASO cytogram. Two clusters are present: basophils (red) and total leucocytes (azure). (C) NRBC cytogram. Azure cluster is formed by total white blood cells. nRBC when present, as in this case, forms a fuchsia cluster oriented along the added white line. In all three cytograms, the additional blue clusters are formed by noise that do not participate in cellular enumeration. FL, fluorescence; FS, forward laser scatter; SS, side laser scatter.

comparison of media and median values between different groups. Shapiro-Wilk test had been previously used to verify the value's distribution. Statistical significance was set at $p<0.05$. Results were reported as a median value with 95% CI for each endpoint: basic, T0, T120 and T240. Sensitivity (SN) and specificity (SP) as well as the agreement (AG) between the cluster's morphology of NRBC cytogram compared with nRBC count after yeast addition were evaluated with receiver operating characteristics (ROC) curve by using specific cut-off identified by means of ROC curves analysis. Statistical analysis was performed using Analyse-it software V.3.90.1 (Analyse-it software, Leeds, UK).

RESULTS

C. albicans suspension

All the subcultured *C. albicans* passed the preliminary phagocytic test and are grown quickly in the BACTEC Peds Plus F culture vials (median of start of the growth curve 12 hours, 23 min; range 11:00–14:35). Gram stain excluded the presence of bacterial contamination and the cultures confirmed the presence of *C. albicans* only. Cytographic features before the mixing with the samples were also evaluated. In DIFF channel, *C. albicans* forms a kind of cloud in an intermediate zone normally free of significant events between NE and lymphocytic clusters; in the BASO channel, a plume of yeast extends along the FS axis, while in NRBC channel, a cluster is present in the same zone usually occupied by circulating nRBC.

Samples cells counting

In table 1, quantitative data and the significance of their differences are shown for groups A, B and C.

In the samples of group A, significant differences were observed for total WBC and NE between basic and T240 counting ($p<0.001$ and $p<0.01$, respectively). On the contrary, the addition of *C. albicans* immediately increased the BA and nRBC counting so much that their differences were highly significant just from the T0 counting ($p<0.0001$ for both). Regarding the PLT however, differences from basic, T0, T120 and T240 counting were not significant.

In the 21 samples of group B, the addition of *C. albicans* caused a statistically significant increase in nRBC values from basic, T0, T120 and T240 counting ($p<0.0001$ for basic to T0 and to T120; $p<0.01$ for T0 to T240).

In the 70 samples of group C quantitative differences of CBC and LDC after yeast addition were negligible in comparison to the basic count (data not shown). On the contrary, nRBC counts change from negative to positive in 63 out of 70 samples. In these 63 samples, the median value of nRBC was

Table 1 Median values and 95% CI of groups A, B and C counting after *Candida albicans* addition

Parameters	Basic median value (95% CI)	T0 median value (95% CI)	T120 median value (95% CI)	T240 median value (95% CI)
Group A: 51 samples without nRBC				
WBC ($\times10^9$/L)	8.29 (7.8 to 9.19)	9.10 (8.33 to 9.85)	9.37 (8.36 to 9.99)	10.05* (9.60 to 10.73)
NE ($\times10^9$/L)	6.20 (5.19 to 6.96)	6.58 (5.99 to 7.45)	7.11 (5.67 to 7.65)	7.41† (7.00 to 8.13)
BA ($\times10^9$/L)	0.03 (0.03 to 0.04)	0.11‡ (0.10 to 0.12)	0.14‡ (0.10 to 0.19)	0.16‡ (0.12 to 0.21)
PLT ($\times10^9$/L)	228 (200 to 253)	223 (208 to 237)	228 (210 to 252)	233 (221 to 273)
nRBC ($\times10^9$/L)	0.000 (0.000 to 0.000)	0.559‡ (0.311 to 0.914)	0.440‡§ (0.336 to 0.568)	0.271‡§ (0.219 to 0.339)
Group B: 21 samples with nRBC				
nRBC ($\times10^9$/L)	0.140 (0.035 to 0.250)	0.927‡¶ (0.271 to 1.178)	–	0.256†¶ (0.179 to 0.707)
Group C: 70 samples with low amount of yeasts added				
nRBC ($\times10^9$/L)	0.000 (0.000 to 0.000)	0.123‡ (0.107 to 0.160)	–	–

*Median value significantly different respect to basic median value by Steel-Dwass-Critchlow-Fligner test with $p<0.001$
†Median value significantly different respect to basic median value by Steel-Dwass-Critchlow-Fligner test with $p<0.01$.
‡Median value significantly different respect to basic median value by Steel-Dwass-Critchlow-Fligner test with $p<0.0001$.
§Median value significantly different respect to T0 median value by Steel-Dwass-Critchlow-Fligner test with $p<0.0001$.
¶nRBC plus pseudo-nRBC.
BA, basophils; NE, neutrophils; nRBC, nucleated red blood cell; PLT, platelets; WBC, white blood cells.

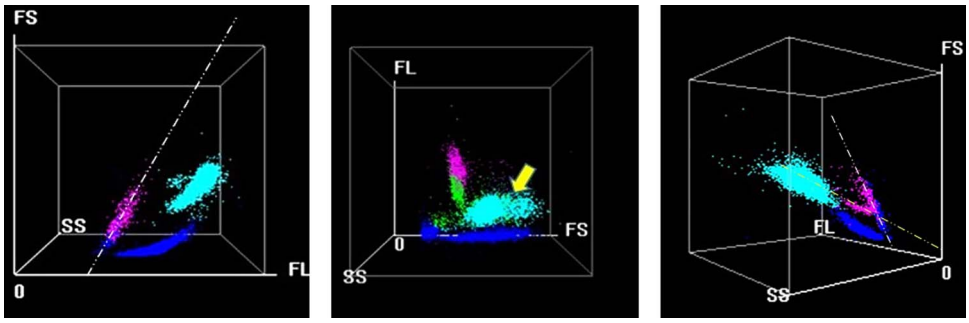

Figure 3 Cytographic changes NRBC and DIFF channels after yeast addition. On the left: NRBC channel immediately after *Candida albicans* addition (T0) (final concentration equal to 1.19×10^9/L): *C. albicans* are counted as nRBC. The corresponding fuchsia cluster has a different shape and its major axis starts far from origin of Cartesian axes with respect to 'true' nRBC (as shown in figure 2). On the centre (same sample): DIFF cytogram (90° rotated) 240 min after yeast addition. The azure cluster is increased in size and extended along the FS axis (arrow) because neutrophils that have swallowed up one or more blastospores increased their volume. On the right: NRBC cytogram (after right rotation) immediately after *C. albicans* addition (0.350×10^9/L) to a sample initially containing nRBC (0.214×10^9/L); two clusters are present: the 'pseudo'-nRBC cluster (white line) formed by blastospores has a shape and orientation clearly different from the 'true' nRBC cluster (yellow line). FL, fluorescence; FS, forward laser scatter; SS, side laser scatter.

equal to 0.123×10^9/L (95% CI 0.107 to 0.160) and the differences with respect to the basic count were significant ($p<0.0001$).

Cytographic evaluation

Before yeast addition, all 142 samples had no anomalies in DIFF, BASO and NRBC cytograms. An nRBC cluster was present only in the group B samples. The most relevant differences in the NRBC and DIFF cytograms after yeast addition to the group A and group B samples are shown in figure 3. In 63 out of 70 of the group C samples, the addition of low amount of *C. albicans* caused a pseudo-nRBC cluster. In figure 4, the 'conclusive' and 'not-conclusive' morphologies for yeast presence are shown.

By using as cut-off a pseudo nRBC count higher than 0.12×109/L compared to cluster's morphology, SN, SP and AG were equal to 1.00, 0.86 and 0.91 respectively. The area under the curve was 0.98 (CI 95% 0.97–1.00; $p<0.0001$) (table 2).

Microscopic review

In all 51 PB smears from group A performed immediately after *C. albicans* addition, extracellular blastospores and pseudohyphae were observed.

In the 25 blood smears that had been prepared at T120 and at T240, numerous NE that phagocytised one or more blastospores were observed. Referring only to total NE, their median value was equal to 6.0% (95% CI 5.5 to 7.0) and 11.0% (95% CI 9.0 to 13.0), respectively. These differences were statistically significant ($p<0.0001$).

DISCUSSION

Circulating yeasts can lead to spurious platelet and WBC counts.[12–14] In addition, pseudo-erythroblastosis has been described.[8 14] These changes were obtained even by adding to PB samples various amount of different *Candida* types to simulate, in vitro, the haematological[7 13] or microbiological[11] features of candidemia. Accordingly, our results showed that the yeast addition causes significant increases of total WBC, NE, BA and nRBC count. BA increases as well as nRBC circulating in PB are present in many clinical situations as inter alia myeloproliferative neoplasms (BA and/or nRBC increase), β-thalassaemia and haemolytic anaemia (nRBC increases only). For these reasons, this data cannot be evocative for BSI by themselves; however, the presence of these cells represents a criterion for microscopic review that could show unexpectedly circulating yeasts in PB especially when they are present in an amount higher than 5×10^{10} CFU/L.[15]

Although spurious increases have been described in presence of circulating yeasts, PLT's count in BC-6800 Analyzer was not influenced significantly. This fact is not surprising because the

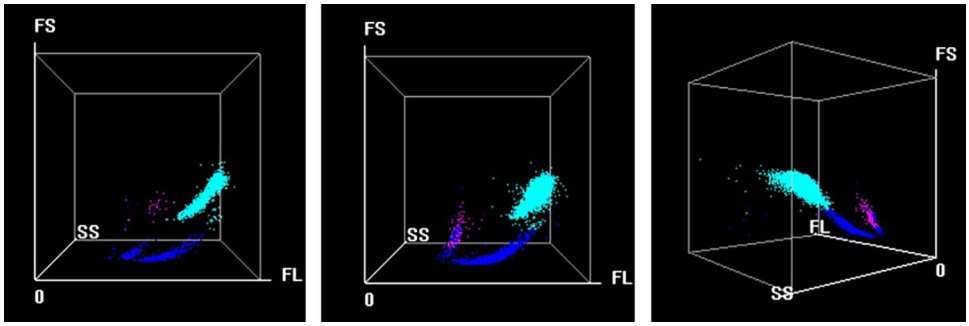

Figure 4 Morphology of the NRBC cluster caused by a low amount of *Candida albicans*. On the left: after yeast addition (final concentration equal to 0.034×10^9/L), a small dispersed cluster is present that does not have a definite shape and orientation. This aspect was considered as 'not conclusive' for yeast presence. On the centre: after yeast addition (final concentration equal to 0.154×10^9/L), the pseudo-nRBC cluster has shape and orientation different from the true nRBC cluster. This aspect was considered as 'conclusive' for yeast presence. On the right (same sample): the peculiar morphological features of the pseudo-nRBC cluster can be better appreciated after rotation. FL, fluorescence; FS, forward laser scatter; SS, side laser scatter.

Table 2 ROC analysis of nRBC values after yeast addition <0.200×10^9/L

	AUC (95% CI) p value	Cut-off (×10^{9}cells/L)	Diagnostic agreement	Sensitivity	Specificity
nRBC	0.985 (0.966 to 1.005), p<0.0001	≥0.120	0.91 (6 false positive samples)	1.00	0.86

AUC, area under the curve; nRBC, nucleated red blood cell; ROC, receiver operating characteristics.

amount of added *C. albicans* that ranged between 0.20×10^9 and 4.0×10^9/L was too low in changing the PLT counting significantly that in our samples was initially equal to 228×10^9/L (median; 95% CI 200 to 253).

On the contrary, morphological changes of BC-6800 cytograms were the most evocative features to suspect the yeasts presence on PB. In particular (figures 3 and 4),

1. the modified and additional NE cluster in DIFF cytograms that are formed by NE containing one or more blastospores.
2. the nRBC cluster in the homologous channel. This cluster appears after *C. albicans* addition immediately and it is reasonable to affirm that it is formed by extracellular yeasts and to name it as 'pseudo'-nRBC cluster. Accordingly, this cluster's morphology is clearly different from true nRBC even when it is caused by a few amounts of circulating yeasts as well described by ROC curve analysis.

Changes of DIFF and NRBC cytograms of BC-6800 well describes the presence of *C. albicans* in PB and seem to be related to pathophysiological events in the case of BSI. In fact, as microscopically observed both in the freshly prepared mixture of blood with *C. albicans* and on the slides review, the phagocytic activity of NE starts with a phase of contact NE/blastospores followed by the emission of one or two pseudopodia immediately.[16] The engulfment process lasts an average of approximately 5 min for yeast and continues for 20–30 min until 4–5 or more of those pass into the NE (figure 1). As a consequence, NE progressively increase in volume and NE cluster in DIFF cytogram was modified.

In the same time, the pseudo-nRBC cluster formed by extracellular yeast becomes progressively less dense due to the blastospores subtracted by the phagocytic activity of the NE that increases from T0 to T240 as observed microscopically.

Two additional diagnostics aspects must be underlined. The former is: in cases of the contemporary presence of a true nRBC, these do not interfere in the recognition of the pseudo-nRBC cluster because their shapes are similar but well distinguishable (figure 3). The latter is: depending on its peculiar morphology only small amounts of *C. albicans* (less than 0.2×10^9 cells/L) are sufficient to recognise a cluster in the NRBC channel as a yeast cluster.

CONCLUSIONS

In our research, instrumental and morphological observations have been performed not only immediately but even up to 4 hours after yeast addition. So, interferences caused by countable particles and the presence of phagocytic cells have been studied. In this way, we could observe that microscopic features observed in the "in vitro" Candidemia are not different from those reported in case of in 'in vivo' BSI regardless of the type of *Candida*.[4 5 8 17 18] Even the correspondence from CBC and LDC on simulated candidemia as well as on clinical BSI has already been described.[6 7 13 14] In addition, in our experimental conditions *C. albicans* has grown in the BACTEC system just as the clinical materials. So, in vitro candidemia seems to be a reliable model to simulate BSI, although a precise correspondence with in vivo events cannot be truly affirmed.

It was stressed that *Candida* BSI is burdened by a high mortality rate especially when an adequate antifungal therapy is not started. So, it has been proposed that alternative diagnostic methods just as (1→3)-β-D-glucan assay could be used before the starting an empirical antifungal therapy.[19] More recently, a novel method based on the use of the miniaturised magnetic resonance (T2MR and T2Candida) has been described for the detection of *Candida* directly from the patient samples with very high SN.[20]

In this scenario, even the cytographic features of BC-6800 Analyzer, which well-describe the presence of low amounts of extracellular and engulfed *C. albicans* in PB, might represent a specific and sensitive alarm that could suggests the use of more sensitive tests for the shortening of the diagnostic and therapeutic timing. In particular, the possibility that cytographic anomalies and the 'pseudo-nRBC' cluster may be caused by the presence of *C. albicans* in the PB must be confirmed by microscopic review. In cases of positivity of this, the pathologist should suggest to clinicians to perform a blood culture. An empiric antifungal treatment may be considered in selected patients at high risk for fungal BSI only.

Take home messages

- Cytographic anomalies on the BC-6800 Haematological Analyzer can reveal the *C. albicans* presence in peripheral blood (PB).
- Cytographic anomalies on the BC-6800 Haematological Analyzer in case of circulating *C. albicans* in PB must be confirmed by microscopic review.
- When the presence of *C. albicans* is confirmed microscopically, the pathologist should suggest to clinicians to perform a blood culture.

Handling editor Slade Jensen

Contributors ALG, FF and AD contributed to conception and design of the work. PI, BS, LF and DS performed the acquisition and interpretation of microbiological data. ALG, MB and MF performed the acquisition and interpretation of instrumental and haematological data. SB performed statistical analysis. PM and AD provided for preparation and review of work. ALG approved the final version published.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

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