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Prevalence of microbial pathogens in blood cultures: an etiological and histopathological study

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Abstract
Performance of BACTEC 9120 blood culture system was compared with the conventional (manual) broth method in detecting blood cultures of 500 patients suffering from various diseases. The BACTEC system proved to be superior in both yield and speed of detection. Four species of yeasts belonging to 2 genera and 13 species of bacteria belonging to 12 genera were recovered from the patient's blood cultures. The study confirmed the serious role of yeasts present in patient blood, and highlighted the serious pathological effect of their secondary metabolites, including clinical symptoms ending with death, besides the changes in the histological pattern in the internal organs, as liver, kidney and spleen using mice as an experimental animal model.

Keywords: Bacterimia, fungemia, histopathological pattern , mycotoxic metabolites.

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1. Introduction

Blood cultures have became one of the most critically important and frequently performed tests in the clinical microbiology laboratory [1.2,3]. Fungemia and bacteremia are terms that simply represent the presence of fungi or bacteria in the patient blood[4]. Invasion of blood by microorganisms usually occurs by one of two mechanism, drainage from a primary focus of infection *via* the lymphatic system to the vascular system, and direct entry of needles (intravenous drug users, or other contaminated intravascular devices, such as catheters or graft material[3,5]. Various culture systems may be used to recover microorganisms from these specimens, but for optimal recovery, a fully automated system and media that are directly inoculated with the specimens are most desirable [1,6]. Several systems are available for the detection of bacterimia and fungemia, which perform relatively well for the recovery of pathogenic isolates [7,8,9] .

One of such systems is the BACTEC system, which uses non invasive fluorescent technology to detect increases in Co_2 produced by the microbial growth. The performance of such a system is influenced by several factors including the volume of blood samples, the use of resins shaking to increase the recovery of aerobic microorganisms, duration of incubation, and final subculture [10].

The purpose of this study was to evaluate the efficacy of different techniques used for the detection of fungemia and bacteremia namely the conventional method and the fluorescent BACTEC technique. Also, to study and evaluate the harmful effect of fungal secondary metabolites on the internal organs using the experimental animal model.

2. Materials & Methods

This study was performed at El-Kasr El-Eni Hospital and the National Cancer Institute.

2.1.Patient selection

Five Hundred patients with a higher probability of bacteremia and or fungemia were subjected in this study. These patients were suffering from repeated episodes of fever accompanying one of the following diseases, rhematic heart diseases (145), Cancer (45), serious burns (22), hepatosplenomegally (54), chronic renal failure (39), post operative, surgery (31), car accidents (2), neonatal sepsis (59) and the rest of cases diagnosed as fever of unknown etiology.

2.2. Sampling

skin was disinfected with 70% ethanol followed by 2% povidine iodide for 2 minutes, then 20ml of blood were withdrawn from each patient via vein puncture using a sterile syringe, in case of neonates only 1-2ml of blood were withdrawn. Each blood sample was equally distributed among the study bottles (conventional broth culture bottle, BACTEC blood culture for aerobes, anaerobes and fungi), to achieve both equal volumes of blood and equal blood-broth ratio.

2.3. Processing

Conventional broth bottles inoculated with patients blood were incubated at 37^0C for fungal, aerobic and anaerobic bacterial growth. The bottle were visually examined in the first 18-24 hours and these showing evidence of microbial growth, such as hemolysis, turbidity, gas bubbles or the presence of discrete colonies on the surface of the sediment red cell layer were examined by direct microscopic smears and sub-cultured on Sabourauds dextrose agar (SDA), brain heart infusion blood agar and 5% sheep blood agar, Mackonky agar and chocholate agar. For those without macroscopic evidence of growth blind subcultures were done, if the subculture was negative, it was repeated after 24 hours then, daily from day 3 to 7, and if still negative a terminal subculture and smear stain examination was done at the end of the 14^{th} day.

For the automatic BACTEC (9120) (Becton Dickinson) system: all inoculated vials were entered into the instrument. They were automatically tested every 10 minutes (positive vials were determined and appeared on the computer screen). The positive bottles were subcultured and Gram smear were prepared, at the end of the testing period, terminal subculture was performed for the negative bottles. [11].

2.4. Identification of the isolates

Yeast isolates were purified and identified using the conventional morphological and biochemical tests [12,13]. Bacterial isolates were purified and identified following the laboratory procedures after Baily and Scott [14].

2.5.Additional investigations

Indirect immune- fluorescence assay (IFA) following the method of Peter and Dawkins [15]. Screening of the yeast 2^{ry} metabolites toxicity using the brine shrimp bioassay method of Bennett [16] .Testing the histopathological effect of yeast 2^{ry} metabolites.

The isolated yeasts were grown on SDA plates for 24 hours, then spore suspension was made by transferring yeast inoculums to sterilized test tube with phosphate buffered saline and the entration was adjusted for all yeasts to 10^3/ml. One ml of each tube was inoculated into 250ml sterilized conical flask containing 100ml Sabouraud dextrose broth, incubated at 37^0C for one week then each flask was centrifuged at 5000 rpm for 5 minutes, filtered using bacterial filters under aseptic conditions. The sterilized filtrate was kept for pathogenicity (toxicity) studies in experimental mice.

Male albino mice weighing 28-30gm each (obtained from animal housing division, National research center, Cairo, Egypt) was kept in cages in 5 groups, each of 7 mice, with continuous supply of food and water. Mice of the first 4 groups were intravenously injected with 0.2ml of the yeast 2^{ry} metabolites (infliltrate) of the isolated yeasts *Candida albicans*, *C. glabrata*, *C. tropicalis* and *Trichosporon beigelii*, respectively. While the fifth group was intravenously

injected with 0.2ml of sterilized uninfected S.D broth as a control group. Mice were continuously observed over period of 8 weeks for the development of any clinical symptoms appearing as a result of infiltrate inoculation. As soon as clinical symptoms appeared, it was recorded and the mouse was sacrificed while the remainders were sacrificed at the termination of the experiment (8 weeks).

2.6. Histopathological work

Liver kidney and spleen of all sacrificed mice were pathologically examined after dissection, fixed in 10% formal saline and processed in a routine manner for histopathological examination using H&E stain [17,18].

2.7. Statistical analysis

Statistical analysis was performed between yield of BACTEC and conventional systems, and their speed of detection using T-test for 2 samples assuming equal variances using the Microsoft excel spread sheet office 1997.

3. Results

A total of 283 patients out of 500 studied cases (65.6%) showed a confirmed blood stream microbial infection (Septicemia). The classification of those patients according to medical diagnosis and type of blood stream microbial infection are shown in Table (1). The BACTEC system gave a significant higher yield and faster speed of detection than the conventional method, as 143 samples were detected by the BACTEC only, 48 by the conventional only and 92 samples were detected by both systems (Table 2). Of those 207 (41.4%) for bacteria and 76 (15.21%) for yeasts (Table 2). (Table 3) demonstrates that *Staphylococcus aureus* is the predominant bacterium 52 cases (25.1%). (Table 3) shows that *Candida albicans* is the predominant yeast 60 (78.9%). (Tables 4 and 5) showed the total yield of bacteria and yeasts with various diagnosed diseases and the recovery of bacterial and yeast isolates by the two systems used for the isolation.

Confirmation of the yeast positive cases was carried out using the indirect immune-florescence assay for the detection of antibodies in patients' sera. All the isolated yeast pathogens gave positive result with this technique.

Table 1 . Classification of patients according to medical diagnosis and type of microbial infection.

Type of diagnosis	No. of patient	No. of bacterial infection	%	No. of fungal infection	%	No. of negative growth	%
R.H.D	145	70	48.3%	5	3.4%	70	48.3
F.U.O	62	17	27.4%	12	19.4%	33	53.2
N.N	59	30	50.8%	12	20.3%	17	28.8
Hepatosplenomegaly	54	25	46.3%	7	13.0%	22	70.7
Cancer Patients	45	11	24.4%	29	64.4%	5	11.1
Fungemia/Bacteremia	41	20	48.8%	2	4.9%	19	46.3
C.R.F	39	17	43.6%	2	5.1%	20	51.3
Post operative	31	9	29.0%	3	9.7%	19	61.3
Burnet Patients	22	8	36.4%	2	9.1%	12	54.5
Car accident	2	0	0.0	2	100.0%	0	00.0
Total	500	207	41.40%	76	15.20%	217	43.4

R.H.D: Rheumatic heart disease **;** F.U.O: Fever for unknown origin **;** N.N: Neonates ; C.R.F: Chronic renal failure.

Table 2. Bacterial and Yeast fungal isolates recovered from blood samples of the patients using different system.

Type of diagnoses	Total			BACTEC			Broth				
R.H.D	145	5	70	3	37	40	1	9	10	1	24
F.U.O	62	12	17	7	12	19	0	2	2	5	3
N.N	59	12	30	5	7	12	3	7	10	4	16
Hepatosplenomegaly	54	7	25	3	14	17	0	5	5	4	6
Cancer Patients	45	29	11	15	5	20	6	2	8	8	4
Fungemia/Bacteremia	41	2	20	2	8	10	0	6	6	0	6
C.R.F	39	2	17	2	8	10	0	3	3	0	6
Post operative	31	3	9	3	3	6	0	3	3	0	3
Burnet Patients	22	2	8	2	5	7	0	2	2	0	1
Car accident	2	2	0	2	0	2	0	0	0	0	0
Total	500	76	207	44	99	143	10	39	49	22	69

R.H.D: Rheumatic heart disease **;** F.U.O: Fever for unknown origin **;** N.N: Neonates ; C.R.F: Chronic renal failure.

Table 3. Bacteria and Yeast fungi recovered from blood specimens.

Organism	No. of isolates	%
a)Bacterial isolates	207	41.4
Staphylococcus aureus	52	25.1
Pseudomonas spp.	41	19.8
Klebsiella spp.	33	15.9
Escherichia coli	28	13.5
Proteus spp.	13	6.3
Enterobacter spp.	8	3.9
Streptococcus viridans	7	3.4
Brucella spp.	6	2.9
Citrobacter spp.	6	2.9
Non haemolytic streptococci	5	2.4
Acinetobacter spp.	4	1.9
Salmonella spp.	2	1.0
Serratia spp.	2	1.0
b)Fungal isolates	76	15.2
Candida albicans	60	78.9
Candida tropicalis	7	9.2
Candida glabrata	5	6.6
Trichosporon beigelii	4	5.3

Table 4. Total yield of yeast and bacteria recovered form blood samples of patients with various diagnosed underlying disease.

Type of diagnoses	Total fungi	*C.albicans*	*C.tropicalis*	*C.glabrata*	*T.beigilii*	Total bacteria	*Staphylococcus aureus*	Non h.streptococct	*Streptococcus viridans*	*Pseudomonas spp.*	*Klebsiella spp.*	*Escherichia coli*	*Proteus spp.*	*Enterobacter* spp.	*Brucella spp.*	*Citrobacter spp.*	*Acinetobacter spp.*	*Salmonella spp.*	*Serratia spp.*
Cancer Patients	29	21	2	5	1	11	3	-	1	2	2	2	-	1	-	-	-	-	-
Neonates	12	12	-	-	-	3	15	-	1	-	4	5	-	5	-	-	-		-
Fever of unknown origin	12	8	3	-	1	17	2	1	-	2	3	2	2	-	2	1	1	-	1
Hepatosplenomegaly	7	6	1	-	-	25	7	-	2	5	5	3	2	-	1	-	-	-	-
Rheumatic heart disease	5	3	-	-	-	70	12	4	2	12	13	8	9	-	3	4	3	-	-
Post operative	3	5	-	-	-	9	4	-	-	5	-	-	-	-	-	-	-	-	-
Chronic renal failure	2	3	-	-	1	17	3	-	-	5	3	4	-	2	-	-	-	-	-
Fungemia/Bacteremia	2	1	-	-	1	20	4	-	1	8	3	2	-	-	-	-	-	2	-
Car accident	2	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Burnet Patients	2	12	-	-	-	8	2	-	-	2	-	2	-	-	1	1	-	-	1
Total	76	60	7	5	4	207	52	5	7	41	33	28	13	8	6	6	4	2	2
%		78.9	9.2	6.6	5.3		25.1	2.4	3.4	19.8	15.9	13.5	6.3	3.9	2.9	2.9	1.9	1.0	1.0

Table 5. Total yield of yeast and bacteria isolated by the BACTEC and conventional system and their approximate speed of detection.

Yeast	Tot.	Bact & conv	Bac. Only	Conv. Only	P.	Speed (hours) Bactec	Conv.	P.
C.albicans	60	20	32	8		38.3	63.1	
C.tropicallis	7	-	6	1		41.6	67	
C.glabrata	5	1	3	1		54.1	117.5	
T.beigelii	4	1	3	-		73.2	123.1	
Total yeast number	76	22	44	10	significant			highly significant
(%)	26.9	7.8	15.6	3.5				
Gram + ve bacteria								
S. aureus	52	21	18	13		21.3	47.1	
Non.H.streptococci	5	3	1	1		27.7	51.3	
Steptococcus sp.	7	12	2	3	P= 0.021 (p 0.05)	25.3	49.1	P= 0.005
Gram-ve bacteria								
Pseudomans sp.	40	14	19	7		17.1	38.7	
Klebsiella sp.	33	11	18	4		18.3	51.3	
Escherichia coli.	29	8	15	6		19.7	43.1	
Proteus sp.	13	5	8	-	t.stat 2.4	16.3	51.9	t.stat -3.008
Enterobacter sp.	8	3	3	2		29.7	43.2	
Brucella sp.	6	-	6	-		91.7	-	
Citrobacter sp.	6	1	3	2		19.3	41	
Acinetobacter sp.	4	2	2	-	Dr=32	23.1	49	Dr=29
Salmonella sp.	2	-	2	-		21.9	-	
Serratia sp.	2	-	2	-		17.1	-	
Total bacteria	207	70	99	38				
(%)	73	24.5	55	14.5				
Total	283	92	143	48				

3.1.Histopathological studies

A screening mortality test of yeasts crude toxin using Brine shrimp toxicity test was carried out before applying the animal experimental studies.

3.2. Mouse bioassay

In vivo toxicity test of the yeast crude toxin, intraperitoneal injection of 1ml crude toxin of each of studied yeast proved to be severely lethal for the inoculated mice while the intraperitoneal mouse inoculation with 0.5ml crude toxin for each of the recovered yeasts separately showed a reduction in severity and longer survival times expressed in minutes post inoculation. The result of this toxicity test revealed the following descending tolerance pattern. Inoculation with 1ml caused death to all mice after few seconds with *C. albicans*, 1-2 min with *C. glabrata,* 2-3 min with *C. tropicalis* and 13-15 min with *T. beigelii*. While the inoculation with 0.5ml caused death to all mice within 3-4 min with *C. albicans,* 7-10 min with *C.glabrata*, 11-17 min with *C. tropicalis* and 37-43 min with *T. beigelii*. The intraperitoneal inoculation with 1ml and 0.5ml of sterilized medium not inoculated with any of the studied fungi (control) did not show any mortality for any of the injected mice.

Inoculation of groups of experimental mice with 0.2ml of the double diluted crude toxin (intravenously) showed, a variety of clinical symptoms ranging from slight reduction in activity to severe convulsions with accelerated respiration rate ending with paralysis and death in different injected groups of mice according to the yeast species studied (Table 6).

Table 6. Percentage of mortalities and survival times of all mice intravenously injected with 0.2ml of diluted crude toxins of the studied yeast fungi .

Candida spp.	Day of death	No of dead mice	Survivors	% of mortalities	Number of Survivors till end of the experiment (8 weeks)
Candida albicans	3rd	1	6	14.3%	
	6th	1	5	28.6%	0
	8th	1	4	42.9%	
	9th	2	2	71.4	
Candida tropicalis	3rd	1	6	14.3%	
	5th	1	5	28.6%	2
	11th	1	4	42.9%	
	19th	2	2	71.4%	
Candida glabrata	4th	1	6	14.3%	
	7th	1	5	28.6%	3
	11th	1	4	42.9%	
	13th	1	3	57.1%	
Trichosporon beigelii	11th	1	6	14.3%	
	18th	1	15	28.6%	4
	27th		4	42.9%	
	11th	1	6	14.3%	

*All control mice survived till the end of the experiment without mortalities or any abnormal clinical symptoms

A varied percentage of mortalities and survival times were observed in all mice injected with 0.2 of the diluted crude toxin with the highest mortality rate in case of *Candida albicans* crude toxin and the least mortalities and longer survival times was observed in case of *Trichosporon beigelii* crude toxin injection (Table 6) . While the control animals inoculated with 0.2ml of sterilized, non inoculated and diluted medium showed no mortalities or clinical symptoms. Various histopathological changes have been noticed in the livers , kidneys and spleens of all injected mice with 0.2ml of the double diluted crude toxins or 2^{ry} metabolites of the yeasts recovered from patient blood stream are shown in Fig. 1 (A , B , C , D , E , and F).

3.3. Statistical analysis

Correlation analyses revealed non-significant differences considering the microbiological data and a considerable significance considering clinical data. The correlation between positive cases and sex was significant (where number of males exceeded that of the females in both bacterial and fungal infections). The correlation between bacterial and fungal infections towards the different clinical cases (rheumatic heart fever, neonate, hepatomegaly, cancer, bacteremia, fungemia and chronic renal failure) was highly significant While the correlation with other clinical cases (post operative, burning, car accident) was significant, but the correlation with the clinical cases (fever of unknown origin) was non-significant with bacterial infections and highly significant with fungal infection. The correlation between the total tested cases and the total positive cases was significant. The correlation between the tested organisms and the mortality rate in brine shrimp bioassay was significant

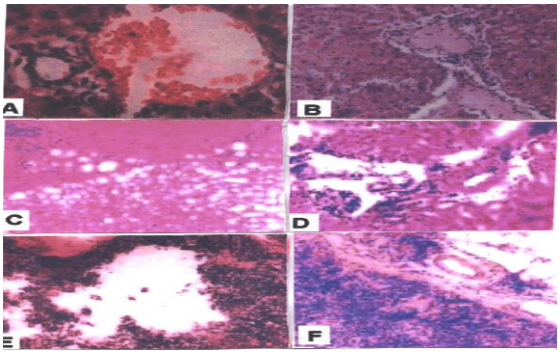

Fig.1. (A)Liver with minimal damage inflammatory infiltration, (B) Liver with necrotic area and consolidation of blood vessels, (C) Kidney showing necrosis and vaculation, (D) Kidney showing necrotic glomerulii and renal tubules, (E) Spleen with severely necrotic area and (F) spleen showing necrosis and fibrosis around an artery.

4. Discussion

The presence of living microorganisms in the patient blood represents one of the most important squealae of infection which constitute a considerable problem as the estimated number of patients with fungemia or bacteremia exceeding millions and are annually increasing with associated mortality ranging from 20 to 50% every year [4, 19].

Yeast infection namely candidiasis is an increasingly common problem in hospitalized patients, with epidemiologic surveys revealing that *Candida* spp. Are now the fourth most common pathogens isolated from the blood of hospitalized patients [7].

The premature infants are at high increasing risk of blood stream infection which may extend to adults suffering from hematological and non hematological malignancy. Diabetes mellitus, renal failure requiring dialysis, hepatocirrhosis, immune deficiency syndrome and loss of skin barrier as in serious burns, skin ulcers and surgery of all types. Also the long term uptake of certain. Medications such as corticosteroids, cytotoxic drugs used for chemotherapy of cancer patients, alter the immune functioning of the blood and increase the risk of blood stream microbial Infection [20].

In the present study the BACTEC (9120) blood culture system was superior to the conventional system regarding the number of isolates detected by each system (143 for BACTEC,49 for broth and 91 for both) and the time of recovery of organisms. This was attributed to the effect of continuous agitation in BACTEC system that proved to increase the yield and speed of detection as reported by other investigators [21,22]. Although the BACTEC system was superior to the conventional in both yield and speed of detection, it has a major defect which should be considered. The presence of false positive radiometric reading which result in an overall load of work and increased time spent for the confirmation of such false positive cultures. While the main disadvantage of the conventional method is the great number of the false positive cultures compared with the BACTEC system. In addition, the delay for 18-24 hours or longer and the need for repeated blind subcultures and microscopic stains. Even after such delay, culture may fail to yield growth. These finding were also concluded by workers [4,5].

Out of the 207 cases with bacterial growth 52 (25.1%) were due to *Staphylococcus aureus*, while 60 (78.9%) cases were due to *Candida albicans* out of the 76 cases with fungal growth. These results were in agreement with those of Silva *et al*.[23] who found that *Candida*

albicans represented 48.1% and other *Candida* spp. were of lower occurrence. He also reported that *Cryptococcus neofermans* was recovered in 10 of invasive fungal infection (blood) besides *Saccharomyces cervisia*e and *Trichosporon mucoides* as two emerging pathogens were also isolated.

Numerous bacterimia and candidemia studies have documented the increasing importance of invasive microbial infection of blood stream of various groups of patients parallel to those included in the present study (renal failer [24] premature neonates [25,26] in immunocompromised patients [27] ; in bone marrow transplantation [28] and in cancer patients [29].

A critical epidemiological study was conducted in Spain (2002-2003) as a population – based surveillance for *Candida* blood stream infections to determine its incidence, the extent of anti fungal resistance, and risk factors for mortality. They defined early mortality as occurring between days 3 to 7 after candidemia and late mortality as occurring between days 8 to 30. They detected 345 of candidemia, for an average annual incidence of 4.3 cases/100.000 population. Overall mortality was 44% . *Candida albicans* was the most frequent species (51% of cases) followed by *C. parapsilosis* (23%), *C. tropicalis* (10%), *C. glabrate* (8%), *C. Krusei* (4%) and other species (3%) [30]. Also a long-term nation wide prospective candidemia study was conducted in Norway (1992-2003). They reported that, the average annual incidence varied markedly between age groups. The incidence was high in patients aged <1 year and in patients age > 70 years. Four *Candida* species *C. albicans* (70%), *C. globrata* (13%). *C. tropicalis* (7%), and *C. parapsilosis* (6%) accounted for 95.5 of the isolates. The species distribution has been constant during the 13-year study period. All *C. albicans* strains were susceptible to fluconzaole. [31].

Fungal (yeast) secondary metabolites might have various toxicological health impacts, however, evaluation of their health effects caused by a great variety of these organic compounds, remained difficult since information about their toxicological impact is lacking [32]. A number of *Candida albicans* antigens can reach the blood stream subject with invasive candidiasis. The molecules investigated include a thermostable protein antigen, believed to be a glycoprotein and a series of metabilites. *Candida* antigens are difficult to study for several reasons, they often form immunocomplexes and may appear in circulation only briefly when the lesion is localized. Mannan has been detected in 47 to 100% of patients with invasive candidiasis depending on the technique used, this antigen is not detected in healthy humans or in patients only colonized or having a superficial infection with *Candida*. The mannan antigen can reach concentration of 1-10 mg/ml in the blood stream [33]. The D-arabitol (five carbon sugar alcohol) which is a metabolite of most pathogenic *Candida* species in vitro as well as in vivo. The endogenous D-arabitol are present in human body fluids such as serum and urine accompanying *Candida* pathogensis [34].

In the present investigation the pathogenic role of such variety of compounds, collectively included in the yeast culture filtrate, (especially those of *C.albicans, C. glabrata and C. tropicallis*) was confirmed with an obvious effects on the viability, and induction appearance of various clinical symptoms ending with mortalities accompanied with destructive changes in the livers, kidney and spleen of the intravenously injected mice. The reports dealing with the pathogenic role of yeasts 2^{ry} metabolites are scanty, however our findings support these reports [35,36].

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