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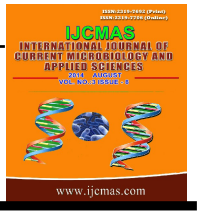

Original Research Article

Bacteriologic and Immunologic Profile of Blood Stream Infected Patients in Intensive Care Unit of Sohag University Hospital, Egypt

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A B S T R A C T

Eighty five patients with suspected sepsis were enrolled in this study during the period from August 2013 to July 2014. The causative organisms were isolated and identified to the species level using API 20 strips as a biochemical identification system. The isolates were tested for antibiotic sensitivity by the disc diffusion method. All isolates were screened for metallo- β –lactamase (MBLs) and extended – spectrum β - Lactamase (ESBL) production by the imipenem/EDTA combined disk test (CDT) and double disc diffusion tests respectively. The percentage of $CD4^+CD25^+$T reg cells and $CD4^+$T helper cells in sepsis patients and in 30 age-and gender-matched healthy subjects as the control group were determined by the flowcytometer to determine the possible increase in Treg number and /or disturbed Treg/T helper cell ratio. Over the study period 68 (80%) patients had sepsis. Gram-negative bacteria were isolated in 60.3% of cases and Gram- positive bacteria in 33.8 % and 5.9% of sepsis cases were caused by candida species. There was a wide spread of multidrug resistance in the isolates. There was suppression of patients' immune response, as demonstrated by the number increase of Treg in patients during sepsis. There was significant increase in the percentage of $CD4^+CD25^+$ Treg cells in the sepsis group (4.8±2.5%) in comparison to the normal control group (1.9±0.7%) with *P*-value < 0.05. Also, the T reg/T helper cell ratio was highly statistically significant higher in sepsis group (0.0956) than in the normal control group (0.0286) with *P*-value < 0.001.

K e y w o r d s

Sepsis;
ICU;
Antibiotic resistance;
Flowcytometer;
T Regs.

Introduction

Nosocomial bloodstream infection (BSI) is a serious and potentially preventable complication of hospitalization. ICU-acquired BSI has been estimated to complicate between 1.2% and 6.7% of all ICU admissions *(Prowle et al., 2011).* According to the American Society of Critical Care Medicine, sepsis is a medical term defined as systemic inflammatory response syndrome (SIRS) in response to an infection *(Johnson et al., 2007).* Infection can be suspected or proven if culture, stain, or polymerase chain reaction (PCR) tests for the specific pathogen are positive, or a clinical syndrome pathognomonic for the infection is present *(Munford and Suffredini,*

2010). A worldwide survey of prevalence and outcomes of infections in intensive care units showed that; among the patients with positive cultures 62% presented gram-negative organisms; 47%, gram-positive; and 19%, fungi *(Vincent et al.,2009)*. In many studies a wide range of bacteria has been described in febrile patients including gram negative bacteria such as *Escherichia coli, Pseudomonas aeroginosa, Klebsiella species, Neisseria meningitidis, Haemophilus influenzae*, and gram positive such as Coagulase negative staphylococci (CoNS), *Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae, and Enterococcus faecium (Rina et al.,2007).* Globally, patients in the ICU have encountered an increasing emergence and spread of antibiotic resistant pathogens *(Radji et al., 2011)*. In the last 20 years since the definition of SIRS/sepsis was proposed, a huge amount of new information has been generated, showing a much more complex scenario of inflammatory and counter inflammatory responses during sepsis. Characterized by an initial intense inflammatory response or "cytokine storm," patients with sepsis may present with fever, shock, altered mental status, and organ dysfunction *(Angus ,2011)*.Then they often develop nosocomial infections with organisms not typically pathogenic in immunocompetent hosts and have reactivation of latent viruses such as herpes simplex virus (HSV) or cytomegalovirus (CMV) (*Hotchkiss et al.,2009).* Although animal studies demonstrate progression to an immunosuppressive phase, epidemiologic studies in clinical sepsis are lacking *(Adib-Conquy and Cavaillon, 2009)*. Recently, accumulating evidence have demonstrated that regulatory T cells (Tregs) play important roles in suppression of immune response, as demonstrated by the number increase and functional enhancement following the onset of severe sepsis *(Jiang et al., 2012)*. It was on this background that; the present study was conducted, which aimed to assess the prevalence of bacterial isolates from nosocomial bloodstream infected patients in the ICU of Sohag University Hospital and their antimicrobial susceptibility pattern and to assess the evidence of immunosuppression in sepsis by determining the role of T regulatory cells as a mechanism that might be responsible for the presumed impaired immunity.

Materials and Methods

Study population

This study was carried out in the Department of Medical Microbiology & Immunology, Faculty of Medicine, Sohag University during the period from August 2013 to July 2014. Blood samples were collected from general surgery ICU patients in Sohag University Hospital. Eighty five patients (Group I) were included in the present study; 47 (55.3%) males and 38 (44.7 %) females with age range from 25 to 57 years, those suspected of developing sepsis after ICU admission. Exclusion criteria were: (a) infection by human immunodeficiency virus (HIV); (b) neutropenia, defined as an absolute neutrophil count <500/mm3; (c) chronic intake of corticosteroids, defined as any daily oral intake of $\geq$ 1 mg/kg of equivalent prednisone for >1 month. In addition to 30 healthy volunteers; 19 (63.3%) males and 11 (36.7%) females in the same age group of patients were randomly selected as the control group (Group II).

Definitions

- We used Center for Disease Control (CDC) definitions of ICU-acquired BSI *(Horan et al., 2008)*. Nosocomial BSI in the ICU was defined as blood cultures

were taken in the presence of clinical evidence of infection for a bacterium or fungus obtained more than 72 hours after admission to the ICU. In accord with CDC guidelines, we did not include cultures of coagulase-negative staphylococci (CoNS) or other common commensal skin organisms as micrococci, corynebacteria, or diptheroids unless two cultures separately isolated the same species of microorganism *(Prowle et al., 2011).*

- Sepsis is diagnosed as finding of SIRS plus documented infection as the cause. SIRS was defined by the presence of at least two of the following criteria: (a) temperature > 38°C or < 36°C; (b) heart rate > 90 beats/ minute; (c) respiratory rate > 20 breaths / minute, arterial partial pressure of carbon dioxide (PCO_2) < 32 mmHg, or mechanical ventilation; (d) white blood cell count > 12, 000 /mm^3. *(American Colleges Of Chest Physicians/Society of Critical Care Medicine Consensus conference 1992 & Johnson et al., 2007)*.
- Multidrug resistance (MDR) was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. Extensive drug-resistance (XDR) was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories). Pan drug-resistance (PDR) was defined as non-susceptibility to all agents in all antimicrobial categories *(Magiorakos et al., 2012)*.

Methods

- A detailed clinical history and detailed physical examination were followed by hematological, biochemical, and other relevant investigations.
- Blood samples were collected aseptically before antibiotic administration and bacterial cultures were done by standard microbiological techniques.
- Patients were considered correctly diagnosed with sepsis if the diagnosis was based on two of the four SIRS criteria shown above in the presence of bacterial infection evidence.

Microbiological analysis

Blood was collected in blood culture bottles containing brain-heart infusion broth. Subcultures were done at 48 hours on Nutrient agar, Blood agar, MacConkey's agar (for primary isolation of *Enterobacteriaceae*), Mannitol Salt agar (for selective isolation and primary identification of *Staphylococcus aureus*), Bile Esculine Azide agar (BEA) (for selective isolation of *Enterococci*), and Cetrimide agar (for selective isolation of *Pseudomonas* species) *(Oxoid Ltd., Basingstoke UK*); and incubated aerobically at 37°C for 24 hours Figure (1). No anaerobic blood cultures were used or evaluated in the present study. When growth was detected, bacterial pathogens were identified by standard microbiological techniques. Isolates were identified by colonial morphology, Gram staining as well as standard biochemical tests. Differentiation of isolates species was done by using API 20 strips *(BioMerieux, France)* as a biochemical identification system.

Antimicrobial Susceptibility Testing

- The susceptibility testing of isolates to different antibiotics was carried out by the disc diffusion method (Modified Kirby- Bauer method) according to the Clinical and Laboratory

Standards Institute (CLSI) guidelines *(CLSI, 2011,a)*.

- The antibiotics included were grouped as follows for testing antibiotic susceptibility pattern of Gram negative isolates: β-lactams (Ampicillin (10 ug), Cefotaxim (30 ug), Ceftazedime (30 ug), Ceftriaxone (30 ug), Cefuroxime (30 ug), and Aztreonam (30μg)); Carbapenems (Imipenem (10μg)); Beta-lactam/inhibitor combination (Ampicillin–sulbactam (20/10 μg)); Quinolones (Ciprofloxacin (5μg)); Aminoglycosides (Gentamicin (10 μg)); Polymixin (Colistin (10μg)), and Extended-spectrum β-lactams (Piperacillin (100 μg)). *(Oxoid Ltd., Basingstoke UK*).
- The antibiotics included were grouped as follows for testing antibiotic susceptibility pattern of Gram positive isolates: β-lactams (penicillin G (10 ug), Oxacillin (1 ug), Cefoxitin (30 ug), Cefotaxim (30 ug)); Beta-lactam/inhibitor combination (Amoxicillin/clavulonic acid (20/10 μg)); Macrolides (Erythromycin (15 ug)); Glycopeptides (Vancomycin (30 ug)); Aminoglycosides (Gentamicin (10 μg)); Trimethoprim-Sulphamethoxazole (25 μg) (Oxoid Ltd., Basingstoke UK).

Phenotypic Detection of metallo- β – lactamase (MBL) Producing Isolates:

The isolates were confirmed as MBLs producers by imipenem/EDTA combined disk test (CDT). To make 0.5 M Ethylenediaminetetraacetic acid (EDTA) solution was prepared by dissolving18.6 g of disodium EDTA.2H_2O was dissolved in 100 mL of distilled water, and the pH was adjusted to 8 by sodium hydroxide (NaOH). Then the mixture was sterilized in the autoclave to prepare a sterile EDTA solution. To prepare EDTA-containing imipenem disks (930 μg EDTA); 5 μL of EDTA solution was added to imipenem disks (10 μg imipenem). These disks were dried immediately in a 37°C incubator and stored at 4°C in airtight vials until use. For each isolate, 1 imipenem disk and 1 EDTA-containing imipenem disk were placed on a suitable distance on the surface of Mueller-Hinton agar plates inoculated with a bacterial suspension equivalent to 0.5 McFarland Standard. After 24 hours of incubation at 37°C, an increase of ≥7 mm in the zone diameter of EDTA-containing imipenem disk compared to imipenem disk was considered to be a positive test for the MBLs producer imipenem resistant strains *(Saderi et al., 2010).* Figure (2)

Phenotypic Detection of extended – spectrum β - Lactamase (ESBL) Producing Isolates

The production of ESBL was assayed using the double disc diffusion method (DDD), cephalosporin discs and combination discs were tested: {cefotaxime 30 μg (CTX 30 μg), ceftazidime 30 μg (CAZ 30 μg), cefpodoxime 30 μg (CPO, 30 μg), cefotaxime 30 μg /clavulanate 10 μg (CTX/CA 30/10 μg), ceftazidime 30 μg / clavulanate 10 μg (CAZ/CA 30/10 μg), cefpodoxime 30 μg / clavulanate 10 μg (CPO/CA 30/10 μg)}. The performance and interpretation were based on the recommendations of Clinical Laboratory Standards Institute *(CLSI, 2010).* The organism was interpreted as ESBL producer if there was an increase of $\geq$ 5 mm in the inhibition zone of the combined disc when compared to the corresponding cephalosporin disc alone according to CLSI guidelines Figure (3).

Phenotypic Detection of MRSA

Confirmed *S. aureus* and CoNS isolates were subjected to cefoxitin disc diffusion test using a 30 μg disc. A 0.5 Mc Farland standard suspension of the isolate was made and lawn culture done on Mueller-Hinton agar plate. Plates were incubated at 37°C for 18 to 24 hrs and zone diameters were measured. An inhibition zone diameter of ≤ 19 mm was reported as oxacillin resistant and ≥20 mm was considered as oxacillin sensitive. (Based on the recommendations of Clinical and Laboratory Standards Institute) *(CLSI, 2011, b).*

Flowcytometry analysis

Peripheral blood samples were drawn in tubes containing EDTA from all the study population (the sepsis group and the normal control group) for Flowcytometric analysis of the percentage of $CD4^+$ helper T cells and $CD4^+CD25^+$ T reg cells among the total lymphocytes. The $CD4^+CD25^+$ T reg/$CD4^+$ T helper cell ratio was evaluated using the nonparametric Mann Whitney U test. A *p*-value < 0.05 was considered significant.

The cells analyzed after staining with monoclonal antibodies to:-

- CD3 (PC5) & CD4 (FITC) for helper T lymphocytes.
- CD4 (FITC) & CD25 (PE) for T regulatory lymphocytes.
- 100 uL of whole blood were placed into polystyrene tubes *(Beckman Coulter, France)* and were subjected to three colours staining with 10 uL/test of fluorochrome conjugated monoclonal antibodies (MoAbs); anti CD3PC5, anti CD25PE and anti CD4FITC antibodies *(Beckman Coulter, France)*. After 20 min incubation at room temperature in the dark, 1.0 mL of lysing reagent *(Versalyse™, Beckman Coulter, France)* was added and lysis was allowed for 10 min at room temperature in the dark. Cells were washed twice with phosphate-buffered saline prior to analysis.
- Cells were analyzed and 10000 events were acquired by *Beckman Coulter Epics-XL flowcytometer* using System II software version 3.0 *(Coulter, USA)*. Membrane intensity of CD3, CD4, and CD25 which is proportional to the number of CD3, CD4, and CD25 epitopes on the cell membrane was estimated in the gated subpopulations by two parameter histograms, and the relative mean fluorescence intensity (MFI) of each sample indicated the amount of MoAb bound to CD3, CD4, and CD25 expressed on peripheral blood cells was recorded. The $CD25^+$ gate was adjusted to contain $CD4^+$ cells that expressed $CD25^+$ than the discrete population of $CD4^+$ cells that does not express CD25.

Statistical Analysis

Statistical differences were determined by using analysis of variance (ANOVA) and Student´s t-test. Results were expressed as mean ± standard deviation of the mean (SD). Qualitative (categorical) data were represented by the number and percent (%). Qualitative data was compared using Chi square test. *P values* < 0.05 were considered significant. *P values* < 0.01 and *P values* < 0.001 were considered highly significant.

Results and Discussion

Over the study period; among 85 ICU admitted patients with suspected septicemia studied; 17(20%) had nonsepsis causes of

SIRS positivity, while 68 (80%) had sepsis; 39 males (57.4 %) and 29 (42.6%) females. Their age range was from 25 to 57 years (Table 1). Forty one cases (60.3%) were caused by gram-negative organisms, 23 (33.8%) were due to gram-positive bacteria, and the remaining 4 cases (5.9%) were caused by candida species (Table 2).

Differentiation of bacterial species

By using API 20 strips *(BioMerieux, France)* as a biochemical identification system; *Escherichia coli* was the most common bacterial isolates accounted for (16.2%) all cases of nosocomial blood stream infection in ICU, followed by CoNS (13.2%); then *Pseudomonas* species (11.8%) *(Pseudomonas aeruginosa* 7.4% (5) and *Pseudomonas luteola* 4.4% (3)), then *Acinetobacter bumannii* and *Klebsiella* species *(Klebsiella pneumonia* 7.4% (5) and *Klebsiella oxytoca* 2.9% (2)) each accounted for (10.3%). *Enterococcus faecalis* accounted for 60% of the enterococcal isolates and *Enterococcus faecium* accounted for 40%. *Enterobacter cloaca* was the least common bacterial isolate (4.4%). (Table 3), (Figure 4)

Antimicrobial resistance pattern of the isolated microorganisms:

Gram negative isolates

Among the gram negative isolates; the highest level of resistance was observed to Ampicillin, Ceftriaxone, Cefotaxime, and Gentamycin, and the lowest level of resistance was observed to Aztreonam, Piperacillin, and Imipenem. *E. coli* were resistant to Ciprofloxacin (45.5%), Gentamicin and Ampicillin/Sulbactam (36.4%).They were highly sensitive to Cefuroxime, Ceftriaxon and Colistin. *Klebsiella* species, *Pseudomonas species*, *Serratia marcescens*, *Enterobacter cloaca*, and *Acinetobacter bumannii* were highly resistant to Gentamycin with resistance rates of (57%), (100 %), (57 %), (100%), and (60 %) respectively. Imipenem and Colistin demonstrated high in vitro activity against at least 90% of strains tested. Only two of the isolates showed resistance to Colistin one was *Pseudomonas aeruginosa* and one was *Klebsiella peumoniae*. For Imipenem; only *E. coli*, *Pseudomonas species* and *Acinetobacter bumannii* were resistant; with resistance rates (9%), (37.3%) and (28.6%) respectively. (Table 4). 20.6% of gram negative isolates were MDR, 11.8% were XDR, and 7.4% were PDR. (Table 5). Production of MBLs enzymes were observed in 12.2% (5/41) of gram negative isolates (3 *Pseudomonas aeruginosa* isolates, and 2 *Acinetbacter bumanii* isolates). Production of ESBL was observed in 41.5% (17/41) of gram negative isolates including; 4 *E. coli*, 7 *A. bummanii*, 3 *K. pneumoniae*, and 3 *P. aeruginosa*. (Table 7).

Gram positive isolates

Staphylococcus species (*S. aureus* and CoNS) were more commonly resistant to Trimethoprim/Sulphamethoxazole (75%) and (66.6%) respectively, cefoxitin and oxacillin (50%) and (44.4%) respectively. (Table 6). The proportion of methicillin resistance among *S. aureus* isolates was 50% (2/4 isolates), No *S. aureus* isolates with reduced susceptibility to vancomycin had been detected. The proportion of methicillin resistance among Coagulase-negative staphylococci isolates was 44.4% (4/9 isolates). Vancomycin resistance was detected in 3(33.3%) coagulase-negative staphylococcal isolates. *Enterococcus* species (*Enterococcus faecalis* and *Enterococcus faecium*) were more commonly resistant to Trimethoprim/ Sulphamethoxazole, penicillin and Cefotaxime (100% for *Enterococcus faecium)*; while for *Enterococcus faecalis*

the resistance rates were 66.7% to Trimethoprim/ Sulphamethoxazole and 50% to penicillin. Two (20%) of enterococcal isolates displayed resistance to vancomycin. Two (50%) of *E. faecium* isolates exhibited resistance to vancomycin while all *E. faecalis* isolates were sensitive to vancomycin (Table 7).

Flowcytometer results

There was a significant increase in the peripheral blood $CD4^+CD25^+$ Treg cells activity as detected by increased number of $CD4^+CD25^+$ Treg cells as a percentage of total $CD4^+$T helper cells in the sepsis group (4.8±2.5%) in comparison to the normal control group (1.9±0.7%); *p value* was < 0.05 (Figure 5 & 6). The percentage of total $CD4^+$T helper cells was decreased in the sepsis group (45.36±12.37%) in comparison to the normal control group (63.40±6.769%); with a highly statistically significant *p value* < 0.001. Also, the median $CD4^+CD25^+$ T reg/$CD4^+$ T helper cell ratio was highly statistically significant higher in sepsis group (0.0956) than in the normal control group (0.0286) with *P*-value < 0.001 (Figure 7), (Table 8).

Table.1 Demographic and clinical characteristics of blood culture positive patients (N=68)

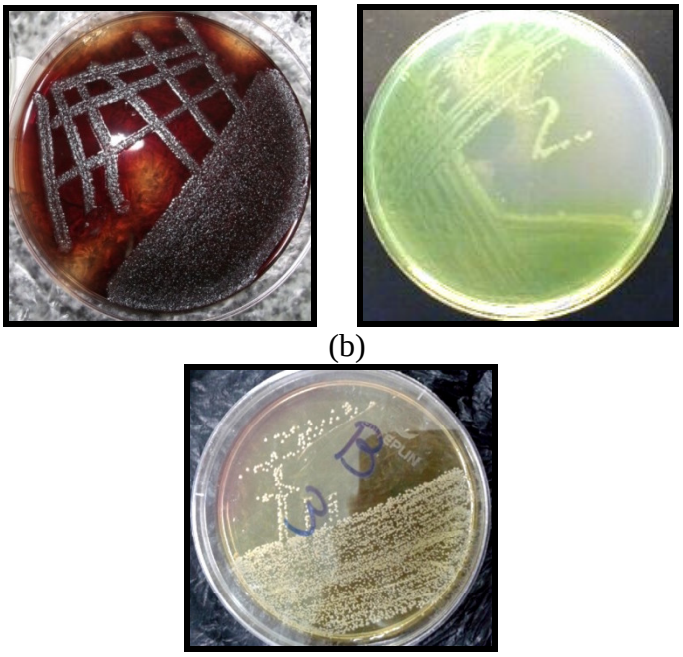
Variable	N	%
Age (y), mean ± SD	41±22.6	
Male gender	39	57.4
Female gender	29	42.6
Severity of illness on the study day		
Sepsis	39	57.4
Severe sepsis	21	30.8
Septic shock	8	11.8
Concomitant diseases	18	26.5
Diabetes mellitus	8	11.5
Chronic obstructive lung disease	7	10.3
Acute renal failure	5	7.3
Devices, used		
Vascular catheters	68	100
Urinary catheter	55	80.9
Nasogastric tube	43	63.2
Invasive mechanical ventilation	41	60.3
Total	68	100

Table.2 Common bacterial pathogens isolated from blood culture (N=68)

Bacterial Pathogen	No.	%
Gram negative bacteria	**41**	**60.3**
Gram positive bacteria	**23**	**33.8**
Candida	**4**	**5.9**
Total	**68**	**100**

Table.3 Distribution of microorganisms isolated from blood cultures

Rank	Pathogen	No. of isolates	Percentage %
1	*Esch. coli*	11	16.2
2	CoNS	9	13.2
3	*Pseudomonas* species	8	11.8
4	*Klebsiella* species	7	10.3
5	*Acinetobacter bummanii*	7	10.3
6	*Enterococcus fecalis*	6	8.8
7	*Serratia marsescens*	5	7.35
8	*Candida* species	4	5.9
9	*Staph. Aureus*	4	5.9
10	*Enterococcus faecium*	4	5.9
11	*Enterobacter cloaca*	3	4.4
Total		**68**	**100**



(a) (b)

(c)

Figure.1 (a) Brown pinpoint colonies of *Enterococcus faecalis* on Bile esculine azide agar, (b) Greenish colonies of *Pseudomonas aeruginosa* on cetrimide agar, and (c) Golden yellow colonies of *Staphylococcus aureus* on Mannitol salt agar

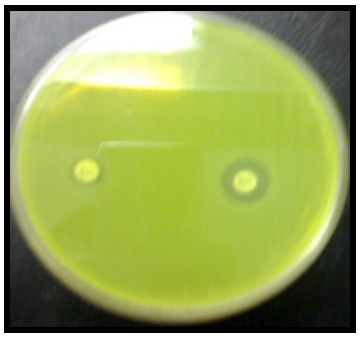


Figure.2 Pseudomonas aeruginosa exhibiting MBL activity by combined disk test showing enhanced inhibition zone of ≥7 mm around IPM + EDTA disc indicating MBL producers.

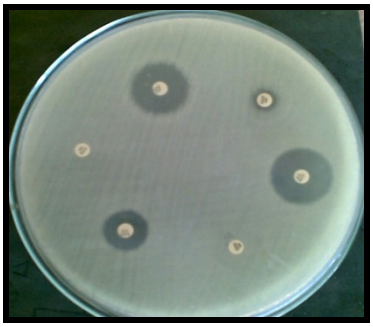


Figure.3 ESBL producing *E. coli* isolate by the double disc diffusion method

Figure.4 (a, b, c &d): API biochemical identification system

(a) API 20 Strept strip with a panel that was identified as *Enterococcus faecalis*.

(b) API 20E strip with a panel that was identified as *Klebsiella oxytocca*.

(c) API 20E strip with a panel that was identified as *serratia marsacens*.

(d) API 20E strip with a panel that was identified as *Enterobacter cloaca*.

Table.4 Antimicrobial resistance pattern of gram-negative bacteria isolated from blood cultures during the study period

The tested antimicrobial	*Escherichia coli* (n=11)		*Klebsiella species* (n=7)		*Pseudomonas species* (n=8)		*Acinetobacter bumannii* (n=7)		*Enterobacter cloaca* (n=3)		*Serratia marcescens* (n=5)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Ampicillin	2	18.2	3	42.8	7	87.5	5	71.4	3	100	4	80
Pipracillin	1	9.1	2	28.6	6	75	4	57	3	100	2	40
Ampicillin/ Sulbactam	4	36.4	3	42.8	6	75	5	71.4	2	66.7	3	60
Cefuroxime	1	9.1	2	28.6	8	100	3	42.8	2	66.7	0	0
Ceftriaxon	0	0	0	0	7	87.5	3	42.8	3	100	0	0
Cefotaxime	0	0	2	28.6	4	50	3	42.8	3	100	0	0
Ceftazedime	3	27.3	3	42.8	3	37.5	4	57	2	66.7	0	0
Aztereonam	2	18.2	0	0	6	75	3	42.8	2	66.7	1	20
Imipenem	1	9.1	0	0	3	37.5	2	28.6	0	0	0	0
Gentamycin	4	36.4	4	57	8	100	4	57	3	100	3	60
Ciprofloxacin	5	45.5	1	14.3	7	87.5	2	28.6	1	33.3	2	40
Colistin	0	0	1	14.3	1	12.5	0	0	0	0	0	0

Table.5 The distribution of resistance pattern among gram-negative isolates

Degree of resistance	No.	%
***Multi-drug resistance**	**14**	**20.6**
Escherichia coli	4	5.9
Klebsiella species	3	4.4
Pseudomonas species	3	4.4
Enterobacter cloaca	2	2.9
Serratia marcescens	1	1.5
Acinetobacter bumannii	1	1.5
***Extensive-drug resistance**	**8**	**11.8**
Klebsiella species	1	1.5
Pseudomonas species	2	2.9
Acinetobacter bumannii	3	4.4
Escherichia coli	2	2.9
***Pan-drug resistance**	**5**	**7.4**
Pseudomonas species	2	2.9
Acinetobacter bumannii	3	4.4

Table.6 Antimicrobial resistance pattern of gram-positive bacteria isolated from blood cultures during the study period

Antibiotic Tested	***Enterococcus fecalis* (n=6)**		***Enterococcus faecium* (n=4)**		***Staphylococcus aureus* (n=4)**		**Coagulase-negative *staphylococci* (CoNS) (n=9)**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Penicillin	3	50	4	100	1	25	5	55.5
Amoxicillin/Clavulanic acid	1	16.6	2	50	0	0	3	33.3
Cefotaxime	0	0	4	100	0	0	2	22.2
Cefoxitin	1	16.6	3	75	2	50	4	44.4
Oxacillin	1	16.6	3	75	2	50	4	44.4
Erythromycin	0	0	3	75	1	25	3	33.3
Gentamicin	0	0	1	25	0	0	1	11
Trimethoprim/ Sulphamethoxazole	4	66.7	4	100	3	75	6	66.6
Vancomycin	0	0	2	50	0	0	3	33.3

Table.7 The distribution of resistance patterns of isolated microorganisms

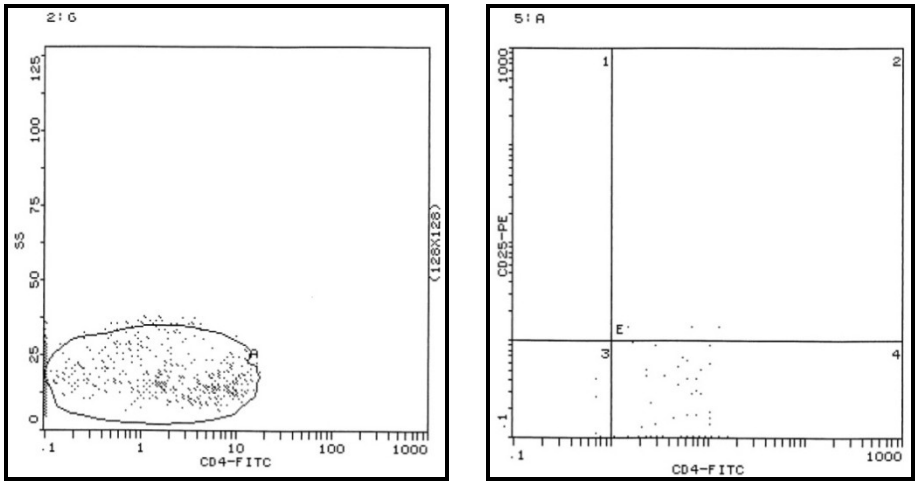
Microorganisms	**No.**	**%**
Total MBLs producers	5	12.2
Pseudomonas aeruginosa, Carbapenem resistant	3	7.3
Acinetobacter baumannii, Carbapenem resistant	2	4.9
Total *ESBL producers*	17	41.5
Acinetobacter baumannii, , ESBL	7	17.1
Escherichia coli, ESBL	4	9.8
Klebsiella pneumonia, ESBL	3	7.3
Pseudomonas aeruginosa, ESBL	3	7.3
Methicillin resistance patterns	6	26.1
Staphylococcus aureus, Methicillin resistant	2	8.7
CoN staphylococci, Methicillin resistant	4	17.4
Vancomycin resistance patterns	5	21.74
CoN staphylococci, Vancomycin resistant	3	13.04
Enterococcus, Vancomycin resistant	2	8.7

Table.8 The median Treg/T helper cell ratio in the sepsis group and in the control group

	Mean rank	**Median of Tregs / T helper cell ratio**	**Mean**	***P*-value**
Sepsis group	17.12	0.0956	0.1011	*P*-value < 0.001 (HS)*
Control group	66.21	0.0286	0.0249	

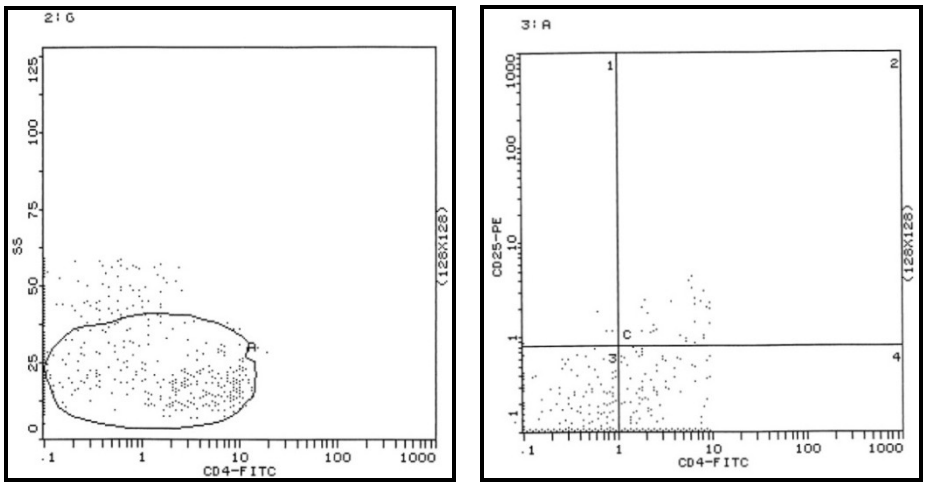
*HS: highly significant

Figure.5 Flow cytometry dot plots. Representative histograms demonstrating the results of a control subject. (a) Side scatter versus CD4 T helper cell (FITC staining).
(b) 2-color flowcytometric analysis, the MFI of CD 25 (PE staining) is indicated on the y-axis, and the MFI of CD 4 (FITC staining) on the x-axis. The upper right quadrant represents the percentage of double-positive cells ($CD4^+CD25^+$ Treg)



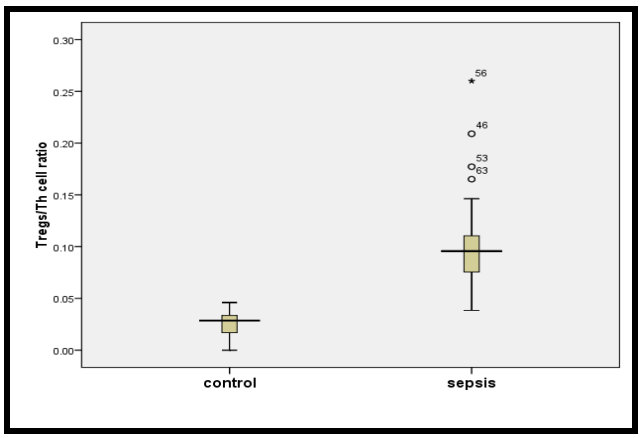
a) **b)**

Figure.6 Flow cytometry dot plots. Representative histograms demonstrating the results of a sepsis patient. (a) Side scatter versus CD4 T helper cell (FITC staining) which show decrease in the percentage as compared to the control. (b) 2-color flowcytometric analysis, the MFI of CD 25 (PE staining) is indicated on the y-axis, and the MFI of CD 4 (FITC staining) on the x-axis. The upper right quadrant represents the percentage of double-positive cells ($CD4^+CD25^+$ Treg); which show marked increase in the percentage as compared to the control.



a) **b)**

Figure.7 The median Treg/T helper cell ratio in the sepsis group and in the control group



The management of infections within the intensive care unit (ICU) poses challenges due to the severity of illness, complex comorbidities, multiple invasive procedures and impaired host defenses characteristic of this patient population. Also, the ICU has even been described as a factory for creating, disseminating, and amplifying antimicrobial resistance *(Brusselaers et al., 2011).* Surveillance of infections and antimicrobial stewardship within the ICU setting are essential pillars of effective health care, not only for the prevention of infection but also for rapid and effective management and conservation of an increasingly limited range of treatment options *(Bonten ,2011).* Despite improvements in therapy, sepsis remains, with high mortality rates, ranging from 22% to 56% *(Salomao et al., 2012).*The range of microorganisms that invade the bloodstream has been studied by several researchers. In our study, Forty one cases of ICU BSI s (60.3%) were caused by gram-negative organisms, 23 (33.8%) were due to gram-positive bacteria, and the remaining 4 cases (5.9%) were caused by candida species. Our results agreed with studies conducted in Saudi Arabia (62.2 and 33.8%) *(Elbashier et al.,1998)* in which gram-negative organisms have been encountered more often from blood cultures than gram-positive organisms. On the contrary, several studies in USA (65 and 25%) *(Wisplinghoff et al., 2004),* Iran (72 and 28%) *(Mamishi et al.,2005)* and UK (66.2 and 31.3%) *(Gray, 2004)* have shown marginally higher prevalence of gram-positive and lower prevalence of gram negative organisms respectively. Different studies have reported different microbiological profile in sepsis patients in ICUs, coagulase negative Staphylococci (27%), S. aureus (15%), and enterococci (10%) have been shown to be the leading pathogens *(Lark et al., 2000).* In our study during the study period, *Escherichia coli* accounted for (16.2%) of all cases of nosocomial BSI in ICU, followed by CoNS (13.2%) and *Pseudomonas* species *(Pseudomonas aeruginosa* (5) and *Pseudomonas luteola* (3)) (11.8%), then *Acinetobacter bumannii* and *Klebsiella* species (*Klebsiella pneumonia* (5) and *Klebsiella oxytoca* (2)) each accounted for (10.3%). *Enterobacter cloaca* was the least common bacterial cause (4.4%). More or less similar observations have been made in cases of bacteraemia in

different countries, however, the proportion and predominance of the organisms varied *(Prakash et al.,2011).* Antimicrobial resistance levels of the isolated organisms were relatively high. 20.6% of gram negative isolates were MDR, 11.8% were XDR, and 7.4% were PDR. 50% of *S. aureus* isolates were Methicillin/Oxacillin resistant *S. aureus* (MRSA) and 44.4% of CoNS were Methicillin/Oxacillin resistant. The antimicrobial resistance levels to Ampicillin, Ceftriaxone, Cefotaxime, and Gentamycin, for the gram-negative bacteria like *E. coli*, *Enterobacter* spp., and *Klebsiella* spp., were relatively high. For Imipenem; only *E. coli*, *Pseudomonas species* and *Acinetobacter bumannii* were resistant; with resistance rates (9%), (37.3%) and (28.6%) respectively. Thus, antibiotic resistance is a major concern for ICUs in our hospital. The lack of culture driven antimicrobial therapy and few infection control practices is largely responsible for the resistant organisms causing BSI in our hospital especially in the ICU. Thus the importance of establishing a microbiological diagnosis, both to ensure that highly resistant pathogens are accurately identified and appropriately treated in this vulnerable ICU population, and to shorten the duration of empirical broad spectrum antibiotic regimens that contribute to the evolution of antimicrobial resistance in our hospital. There is an important point which must be taken into consideration that; despite a vigorous and uncontrollably sustained inflammatory response to infection in septic patients, immune dysfunction is a notable feature of severe sepsis. Immune dysfunction predisposes septic patients to secondary infection that can delay recovery (*Skrupky et al., 2011).* In addition, immune dysfunction may delay restitution of the necessary milieu crucial to healing following severe sepsis and can potentially contribute to ongoing multi‐organ dysfunction. Understanding the mechanisms of immune dysfunction and potential secondary implications are paramount in the management of severely septic patient. Many mechanisms are responsible for sepsis induced immuno-suppression, including apoptotic depletion of immune cells, increased T regulatory and myeloid-derived suppressor cells, and cellular exhaustion *(Boomer et al., 2014).* Understanding of sepsis‐induced immune dysfunction offers vast opportunities for improving the mortality and morbidity from prolonged ICU stays and secondary infections. In this work we tried to understand the potential role of Tregs in the pathophysiology of septic response. The present study analyzed circulating Treg in sepsis patients to investigate whether this cell population is related to infection. The results show that there was a significant increase in the percentage of $CD4^+CD25^+$ Treg cells in the entire sepsis group (4.8±2.5%) in comparison to the normal control group (1.9±0.7%); *p value* was < 0.05. Also, the $CD4^+CD25^+$ T reg/$CD4^+$ T helper cell ratio was calculated both in sepsis group and in normal control group. The median ratio was highly statistically significant higher in the entire sepsis group (0.0956) than in the normal control group (0.0286) with *P*-value < 0.001 which support the concept that regulatory T lymphocytes play a role in the control of immune responses and are affected by injury and sepsis. This may be related to their capacity to interact with components of the innate and adaptive immune responses and to their ability to be activated nonspecifically by bacterial products and/or cytokines *(Venet et al., 2008).* Our results agreed with those of *Monneret et al. (2003)*; who were the first to demonstrate, in a small cohort of septic

shock patients, the presence of an increased percentage of circulating Tregs in blood. These results were confirmed in a subsequent study by this group, which described further that this relative increase was in fact a result of a decrease of the $CD4^{+}CD25^{-}$ circulating T lymphocyte numbers (Treg counterparts) and not so much a change in the absolute Treg count in patients *(Venet et al.,2004).* Our results agreed with those of *Venet et al. (2004)* who said that; an increased percentage of $TCD4^{+}CD25^{+}$ cells (Tregs) among $CD4^{+}$ lymphocytes was found in septic patients compared with healthy individuals because of a decreased proportion of $TCD4^{+}CD25^{-}$cells which was (45.36±12.37%) in the sepsis group in comparison to the normal control group (63.40±6.769%); with a highly statistically significant *p value* < 0.001. This is an evidence supports a role of Tregs in sepsis-induced immunosuppression. However, *Brunialti et al. (2012)* evaluated the presence of Tregs in septic patients and they found that the percentages of Tregs did not differ between patients and healthy volunteers. So; we believe that a better understanding with regard to the mechanism of regulatory function of Tregs, which are found to exert such a profound effect on the immune response, may help improve our clinical ability not only in diagnosis but also in treatment for the critically ill septic individuals.

In the present study most of the pathogens isolated showed high rate of resistance to most commonly used antibiotics. Additional efforts are needed in the future to slow down the emergence of antimicrobial resistance. Our study provides baseline data for future studies on the success of maximizing quality interventions to contain the spread of multidrug resistant organisms and to target antimicrobial therapy more appropriately. Taken together, the available data of our observations suggest that; the manipulation of Tregs might provide a novel treatment modality for septic response.

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