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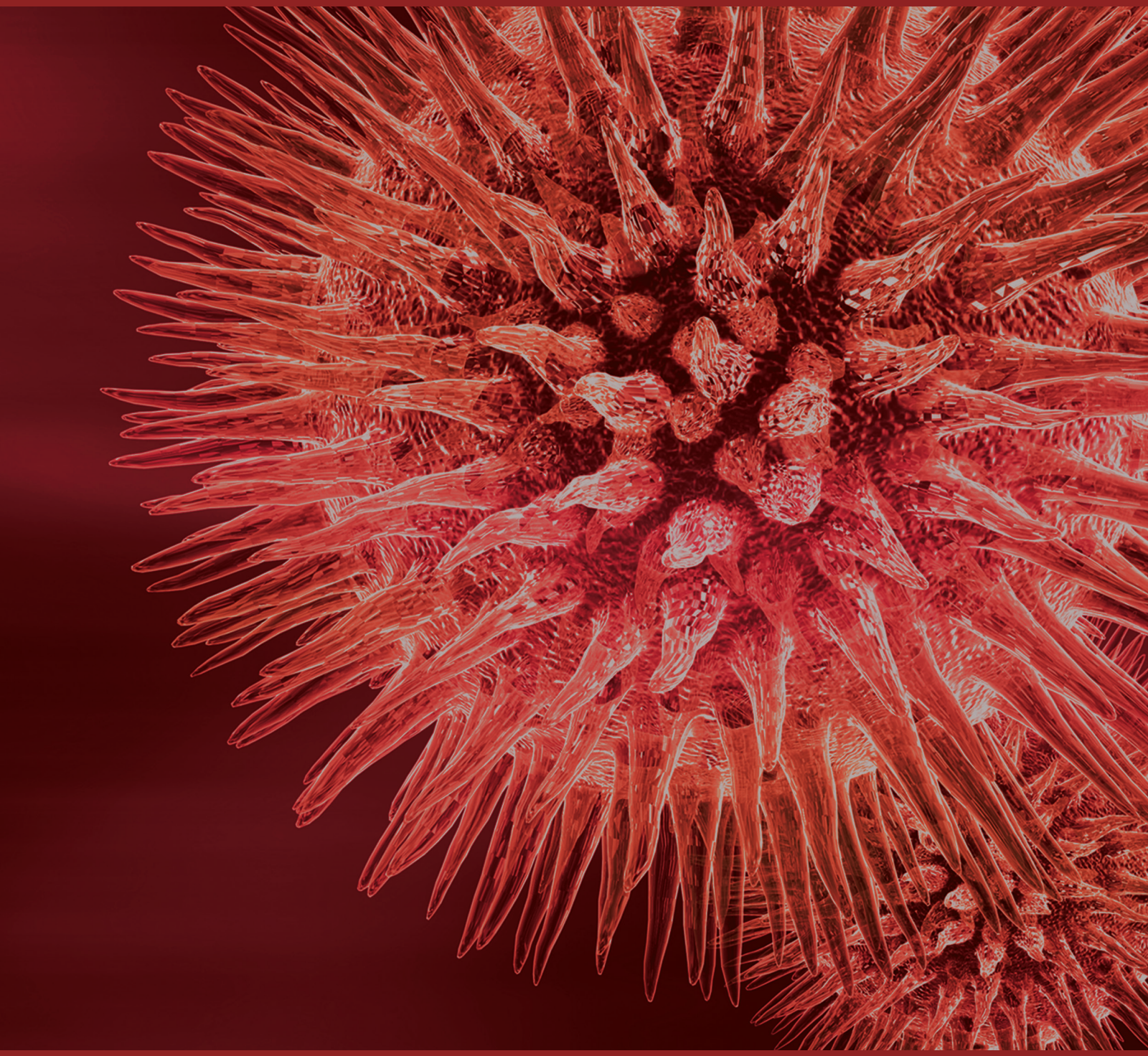
Guest Editors: Renu Bharadwaj, Abhijit Bal, Ketoki Kapila, Vidya Mave, and Amita Gupta



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Editorial

Blood Stream Infections

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Blood stream infection (BSI) is one of the most devastating preventable complications in Critical Care Units. It has far-reaching consequences resulting in prolonged length of hospital-stay, high costs to the individual and exchequer, and, in many instances, loss of life. Although exact rates of BSI differ markedly worldwide, figures in the US are around 19.8 episodes per 1000 central-line days (CI 95%; 16.1–23.6) with an approximate incidence of 100,000 episodes annually [1]; the rate, in USA, falls to 5.8 (CI 95%; 3.8–7.8) when only microbiologically documented episodes are considered [2] and to 8.75% in Indian ICUs [3]. The need to bring these two data groups (microbiologically proven and clinical sepsis) as close as possible is every infectious disease specialist's dream.

Advances in medical science have resulted in increased interventions in critically ill patients creating foci from where bacteria can gain access to the blood stream resulting in an increase nosocomial BSI. They represent about 15% of all nosocomial infections and affect approximately 1% of all hospitalized patients [1]. A hospital-related BSI would deem to have occurred after a patient has completed ≥48 h of stay in the hospital or has a central line for 48 h or more [4]. Community acquired BSIs can also occur. A BSI is primary when the central line is the only probable source of infection and secondary when there is an underlying cause for the BSI (genitourinary/respiratory infection or any other obvious source of infection in the body).

Among the bacterial causes of BSI, *Staphylococcus aureus*, coagulase negative *Staphylococci*, and *Enterococcus faecalis* are the commonest among Gram positive organisms; *Escherichia coli*, *Klebsiella pneumonia*, and *Serratia spp* are the commonest among Enterobacteriaceae; and *Pseudomonas spp* and *Acinetobacter baumannii* are the commonest amongst the nonfermenter Gram negative organisms [2, 5]. Among fungi, it is nonalbicans *Candida* spp followed by *Candida albicans* that are common [6]. However, organisms vary with several factors such as (i) type of health-care facility involved, (ii) presence of a central venous/arterial catheter, (iii) type of catheter used, (iv) duration of catheterization, (v) prevalent organisms in the center, (vi) immune status of the host, (vii) underlying comorbidities, (viii) level of preventive and barrier precautions undertaken, and (ix) initial antimicrobial therapy [1, 7].

The severely immune-compromised patient is prone to fungal as well as bacterial blood stream infections. However, the lack of diagnostic tools for early detection of candidemia and other fungal infections limits the number of studies on this issue. Clinical and radiological signs are nonspecific, and traditional culture-based tools suffer from low sensitivity. Tests that have generated interest include combined detection of mannan and anti-mannan antibodies, β-1, 3, D-glucan detection, and a number of molecular techniques. Unfortunately drawbacks of nonculture techniques include moderate level of sensitivity and specificity and lack of standardization [8].

Currently, multidrug resistant bacteria, residing in ecological niches in hospitals, present therapeutic challenges

when they manifest as bacteremias [7]. Bacteriological profile and drug resistance patterns tend to be peculiar to an institute that is dealing with a special category of patients. In this issue, a tertiary care center in Brazil reports a retrospective cohort study of the prevalence of vancomycin resistant *Enterococcus faecium* from BSI, and K. Gohel et al. report the profile and drug resistance patterns of blood culture isolates from a tertiary nephrourology institute of India. In India, the burden of bacterial infection is estimated to be very high; however, systematic data is limited [2]. Identification of the extent of the problem generates evidence for advocacy for regulation of currently unregulated antibiotics. Additionally, such data guides the policy on implementation of antibiotic stewardship programs and standardized infection control guidelines. Knowledge of the pattern of antibiotic resistance prevalent in severe infections could also motivate and direct new drug discovery.

The need for early aggressive therapy in BSI cannot be overemphasized. The "time-window" for administration of appropriate therapy is <6 h once symptoms are apparent and many agree that the very first hour is critical [9]. An ideal platform must offer quick, specific diagnosis, be economical, and have minimum hands-on time. Use of biomarkers for diagnosis and monitoring of sepsis holds promise. Not only do they distinguish infective from noninfective sepsis, but also the serial use of biomarkers can be used for determining effectiveness of an intervention. Procalcitonin and C-reactive protein are already in use widely but the search is on for even better agents. The performance of soluble triggering receptor expressed on myeloid cells-I (sTREM-1), soluble urokinase-type plasminogen receptor (suPAR), proadrenomedullin (pro ADM), and presepsin appears promising and offers better prognostic performance than procalcitonin [10, 11]. The use of biomarkers in sepsis has been discussed in this special issue. Apart from biomarkers, a mass-spectroscopy based approach known as MALDI-TOF (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) can provide genus and species level identification within minutes enabling significant time saving over conventional methods of identification [12]. Research is on to determine the potential of mass-spectrometry to provide other useful information to the clinician, epidemiologists, and clinical microbiologists, such as genotyping, virulence marker, and resistance mechanism. A modified PCR/ESI-MS (PCR followed by electrospray ionization mass spectrometry) method is now available and holds promise for detection of pathogen directly from clinical samples. This evolving method has been discussed in this special issue. The need of the hour for the technology sector is to work upon such tools that are not only efficient but also economically viable so that developing nations can benefit as, ironically, it is here that the need for such measures is most.

The therapeutic challenges posed by the blood stream pathogens make it imperative that better strategies are developed to prevent infections. Education and training of health-care workers, use of maximum sterile barrier precautions for all patients on central/peripheral lines, and appropriate skin antisepsis during central venous catheter insertions are some simple guidelines that can save precious lives [12]. Medicated catheter-lock solutions and use of antiseptic/antibiotic impregnated central venous catheter and chlorhexidine impregnated sponge dressings are being looked into in various centers [1, 4].

This special issue brings forth various aspects of blood stream infections from around the world, including advances in detection and use of possible alternative pharmaceutical agents. The quest for reducing blood stream infections is gaining momentum worldwide as in most cases, it is eminently preventable. BSI that plagues critical care centers all over the world is a continued challenge, has many formidable frontiers, and remains an enigma even today. We hope this issue will stimulate researchers to work on improving the methodologies for detection, prevention, and management of blood stream infections so that we can reach a stage of "zero" morbidity and mortality from this infection.

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Research Article

Molecular Screening of Virulence Genes in Extraintestinal Pathogenic *Escherichia coli* Isolated from Human Blood Culture in Brazil

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Extraintestinal pathogenic *Escherichia coli* (ExPEC) is one of the main etiological agents of bloodstream infections caused by Gram-negative bacilli. In the present study, 20 *E. coli* isolates from human hemocultures were characterized to identify genetic features associated with virulence (pathogenicity islands markers, phylogenetic group, virulence genes, plasmid profiles, and conjugative plasmids) and these results were compared with commensal isolates. The most prevalent pathogenicity island, in strains from hemoculture, were PAI IV_{536}, described by many researchers as a stable island in enterobacteria. Among virulence genes, *iut*A gene was found more frequently and this gene enconding the aerobactin siderophore receptor. According to the phylogenetic classification, group B2 was the most commonly found. Additionally, through plasmid analysis, 14 isolates showed plasmids and 3 of these were shown to be conjugative. Although in stool samples of healthy people the presence of commensal strains is common, human intestinal tract may serve as a reservoir for ExPEC.

1. Introduction

Escherichia coli is one of the most common microorganisms of the human intestinal microbiota. However, a small percentage of *E. coli* is capable of causing extraintestinal infections (extraintestinal pathogenic *Escherichia coli*—ExPEC), and these ExPECs are considered some of the main etiological agents of bacteremia caused by Gram-negative bacilli [1, 2]. According to the phylogenetic classification, ExPECs typically belong to group B2 and less commonly to group D, whereas commensal intestinal strains belong to group A or B1 [3]. ExPEC pathogenicity is due to the presence of genes, located on plasmids or chromosomes that encode virulence factors. When present on the chromosome, these genes are typically found in specific regions called pathogenicity islands (PAI). Given that the severity of bacterial infections is often due to the genetic features of the pathogenic agent and that few studies have investigated genetic aspects of ExPEC isolates from bacteremia in Brazil [4, 5], the aim of the present study was to characterize 20 *E. coli* isolates from human hemocultures for genetic features associated with ExPEC. The investigation was based on the screening for PAI associated sequences, determination of phylogenetic group, genotypic identification of the major virulence factors of ExPEC, and plasmid analyses, and these results compared with commensal strains. This information may help us better understand the pathogenesis of these bacteria.

2. Materials and Methods

2.1. Bacterial Isolates. To perform the study, 20 *E. coli* strains isolated from human hemocultures were kindly provided by Professor Marilda C. Vidotto (Brazil) [6] and 51 *E. coli* strains were obtained from stools of 19 healthy Brazilians. The strains were stored in brain heart infusion with 20% glycerol at −20°C. The polymerase chain reaction (PCR) was used for the genetic characterization of PAI associated sequences, presence of virulence genes, and for phylogenetic classification. PCR amplicons were visualized on 1.0% agarose gels stained with GelRed (Biotium). After gel electrophoresis

the images were captured using Image Capture Systems (LPixImageHE).

2.2. Detection of PAI Markers. The presence of sequences associated with seven different PAIs, previously characterized in uropathogenic *E. coli* (UPEC), was determined (PAI I_{536}, II_{536}, IV_{536}, I_{CFT073}, II_{CFT073}, I_{J96} and II_{J96}) [7] (Table 1).

This PCR contained 1 U Taq DNA polymerase (Invitrogen) in 2x PCR buffer (Invitrogen), 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, and 20 pmol/μL of each primer (Table 1). The program consisted of 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min [7]. The positive control used in the PCR was J96.

2.3. Phylogenetic Classification. Phylogenetic classification showed that the *E. coli* strains belonged to four groups (A, B1, B2, or D) based on the presence of the *chu*A and *yja*A genes and the DNA fragment (TSPE4.C2). This PCR contained 1.25 U Taq DNA polymerase (Invitrogen) in 1x PCR buffer (Invitrogen), 20 pmol of each dNTP, 2.5 mM $MgCl_2$, and 1 μM of each primer (Table 1). The program of PCR consisted of 94°C for 4 min, followed by 30 cycles of 94°C for 5 seg and 54°C for 10 seg, with a final extension step at 72°C for 5 min [3].

2.4. Virulence Factors Genes. The pathogenicity of *E. coli* is associated with the presence of virulence factors that can be encoded by chromosomal and plasmid genes, and thus 19 genes encoding virulence factors were investigated. The genes selected were specific for hemolysins (*hly*A and *hly*F), cytotoxic necrotizing factors (*cnf*1 and *cnf*2), colicin V (*cva*C), aerobactin (*iut*A), yersiniabactin (*fyu*A), salmochelin (*iro*N), P-fimbriae (*pap*C and *pap*G), S-fimbrial adhesin (*sfa*A and *sfa*S), afimbrial adhesin (*afa*), serum resistance (*iss* and *tra*T), brain microvascular endothelium invasion (*ibe*10), K1 capsule (*kps*II and K1), and ompT outer membrane protein (*omp*T) [8–10]. This PCR contained 1.25 U Taq DNA polymerase (Invitrogen) in 1x PCR buffer (Invitrogen), 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, and 1 μM of each primer (Table 1). The program of PCR consisted of 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min.

2.5. Plasmids Profile. To analyze the plasmid profile, the plasmids of wild strains and the plasmids R27 (110 MDa), JPN11 (66 MDa), PSA (23 MD), and pRK (13, 2 MDa) used as markers of molecular mass were extracted by alkaline lysis [11]; the molecular weight of the plasmids was measured (LabImage 1D software) and the ability to transfer was determined.

The strains that harbored plasmids were chosen for mating experiments. The strains were grown in LB (Luria Bertani Broth) until the exponential phase. 1.2 mL of this culture was transferred to a flask containing 0.4 mL of the recipient culture in the stationary phase, *E. coli* K12-711 [12]. The mixture was incubated at 37°C for 3 hours. Transconjugants resistant to drugs were selected on MacConkey agar containing inhibitory concentrations of nalidixic acid (resistance present in *E. coli* K12-711), tetracycline, ampicillin, or kanamycin. The colonies grown on each selective plate were tested for the presence of virulence genes and pathogenicity islands, according to the virulence pattern of the donor strain. The resistance profile, necessary for this test, is shown in Table 2.

3. Results and Discussion

Extraintestinal pathogenic *Escherichia coli* (ExPEC) is one of the leading causes of bloodstream infections (BSI) worldwide. In Brazil, it was observed a high mortality associated with BSI [13]. Despite the importance of *E. coli* bloodstream infections due to their high morbidity and mortality, the pathogenesis is not well known [14, 15] and not studied enough in South America [4, 5].

In this study we screened 20 strains from bacteremia from newborns (10%), children aged between 6 months and 14 years (20%), and adults (70%) and from two Brazilian teaching hospitals for the determination of phylogenetic group, pathogenicity islands (PAI) associated sequences, and important virulence factors responsible for extraintestinal pathogenesis. The results were compared with those obtained with isolates from the stools of healthy humans. The relationship between the presence of PAIs, virulence genes, and the phylogenetic group was analyzed.

Among the 20 isolates from the hemocultures tested, 70% of the isolates displayed PAI associated sequences (total of 22 islands), while in the commensal strains 52.94% of them displayed PAI (total of 45 islands). In agreement with previously published data [7, 16, 17], PAI IV_{536} was the most prevalent in both groups, following the PAI I_{CFT073} and PAI II_{CFT073}, as shown in Tables 3 and 4. PAI IV_{536} has been described by many researchers as a stable island, and it is one of the most commonly found PAIs in enterobacteria [7, 18]. Our results show that the islands present in UPEC, although poorly researched in septicemic strains, are also found in *E. coli* isolated from hemocultures. This similarity can be associated with the fact that the urinary tract infection is one of the most common infections and bacteraemia is often a complication of this infection. But there are other ways for the presence of bacteria in the blood, such as meningitis and polymicrobial intra-abdominal infections. The presence of bacteria in the bloodstream suggests the ability of these pathogens to survive in an environment with scarce free iron and to resist the bactericidal activity in the blood [4].

It is not clear yet if all *E. coli* from the intestinal tract of healthy people can be considered commensal, as some isolates showed up to five pathogenicity islands. Already it has been reported that ExPEC can asymptomatically colonize the intestinal tract [7].

In this study, genes related to toxin and hemolysin production were researched and included *cnf*2, *cnf*1, *hly*A, *hly*F, and *cva*C. The *hly*A gene is frequently detected in ExPEC and it was the most prevalent gene in our strains of hemoculture (30%) [19, 20]. *Escherichia coli* hemolysin (*hly*A) is a pore-forming bacterial exotoxin that may contribute to

Table 1: Primers for detection of PAIs markers, phylogenetic analysis, virulence genes, and their respective virulence factors.

Genes	Sequence (5′ to 3′)	Size of product (bp)	Virulence factors	Reference
PAI I_{536}	TAA TGC CGG AGA TTC ATT GTC AGG ATT TGT CTC AGG GCT TT	1.800	α-Haemolysin, CS12 fimbriae, and F17-like fimbrial adhesin	Sabaté et al., 2006 [7]
PAI II_{536}	CAT GTC CAA AGC TCG AGC C CTA CGT CAG GCT GGC TTT G	1.000	α-Haemolysin and P-related fimbriae	Sabaté et al., 2006 [7]
PAI IV_{536}	AAG GAT TCG CTG TTA CCG GAC TCG TCG GGC AGC GTT TCT TCT	300	Yersiniabactin siderophore system	Sabaté et al., 2006 [7]
PAI I_{CFT073}	GGA CAT CCT GTT ACA GCG CGC A TCG CCA CCA ATC ACA GC GAA C	930	α-Haemolysin, P-fimbriae, and aerobactin	Sabaté et al., 2006 [7]
PAI II_{CFT073}	ATG GAT GTT GTA TCG CGC ACG AGC ATG TGG ATC TGC	400	P-fimbriae and iron-regulated genes	Sabaté et al., 2006 [7]
PAI I_{J96}	TCG TGC TCA GGT CCG GAA TTT TGG CAT CCC ACA TTA TCG	400	α-Haemolysin and P-fimbriae	Sabaté et al., 2006 [7]
PAI II_{J96}	GGA TCC ATG AAA ACA TGG TTA ATG GG GAT ATT TTT GTT GCC ATT GGT TAC C	2.300	α-Haemolysin, Prs-fimbriae, and cytotoxic necrotizing factor 1	Sabaté et al., 2006 [7]
*chu*A	GAC GAA CCA ACG GTC AGG AT TGC CGC CAG TAC CAA AGA CA	279	Hemetransport in enterohemorrhagic O157:H7 *E. coli*	Clermont et al., 2000 [3]
*yja*A	TGA AGT GTC AGG AGA CGC TG ATG GAG AAT GCG TTC CTC AAC	211	Protein of function unknown	Clermont et al., 2000 [3]
TSPE4.C2	GAG TAA TGT CGG GGC ATT CA CGC GCC AAC AAA GTA TTA CG	152	Putative DNA fragment (TSPE4.C2) in *E. coli*	Clermont et al., 2000 [3]
*kps*II	GCG CAT TTG CTG ATA CTG TTG CAT CCA GAC GAT AAG CAT GAG CA	272	Capsule synthesis K1 e K5	Johnson and Stell, 2000 [8]
K1	TAG CAA ACG TTC TAT TGG TGC CAT CCA GAC GAT AAG CAT GAG CA	156	Capsule K1	Johnson and Stell, 2000 [8]
*cva*C	CAC ACA CAA ACG GGA GCT GTT CTT CCC GCA GCA TAG TTC CAT	680	Colicin V	Johnson and Stell, 2000 [8]
*iut*A	GGC TGG ACA TCA TGG GAA CTG G CGT CGG GAA CGG GTA GAA TCG	302	Aerobactin siderophore receptor	Johnson et al., 2008 [9]
*fyu*A	TGA TTA ACC CCG CGA CGG AA CGC AGT AGG CAC GAT CTT GTA	880	Yersiniabactin	Johnson and Stell, 2000 [8]
*pap*C	GAC GGC TGT ACT GCA GGG TGT GGC G ATA TCC TTT CTG CAG GCA GGG TGT GGC	328	P fimbriae	Le Bouguénec et al., 1992 [10]
*pap*G	CTG TAA TTA CGG AAG TGA TTT CTG ACT ATC CGG CTC CGG ATA AAC CAT	1070	P fimbriae	Johnson and Stell, 2000 [8]

Table 1: Continued.

Genes	Sequence (5′ to 3′)	Size of product (bp)	Virulence factors	Reference
*sfa*A	CTC CGG AGA ACT GGG TGC ATC TTA C CGG AGG AGT AAT TAC AAA CCT GGC A	410	Sfa fimbriae	Le Bouguénec et al., 1992 [10]
*sfa*S	GTG GAT ACG ACG ATT ACT GTG CCG CCA GCA TTC CCT GTA TTC	240	Sfa fimbriae	Johnson and Stell, 2000 [8]
Afa	GGC AGA GGG CCG GCA ACA GGC CCC GTA ACG CGC CAG CAT CTC	559	M fimbriae	Johnson and Stell, 2000 [8]
*ibe*10	AGG CAG GTG TGC GCC GCG TAC TGG TGC TCC GGC AAA CCA TGC	170	Invasion of brain endothelium	Johnson and Stell, 2000 [8]
*hly*A	AAC AAG GAT AAG CAC TGT TCT GGC ACC ATA TAA GCG GTC ATT CCC GTC	1.177	Hemolysin	Johnson and Stell, 2000 [8]
*cnf*1	AGG ATG GAG TTT CCT ATG CAG GAG CAT TCA GAG TCC TGC CCT CAT TAT T	498	Cytotoxic necrotizing factor 1	Johnson and Stell, 2000 [8]
*cnf*2	AAT CTA ATT AAA GAG AAC CAT GCT TTG TAT ATC TA	543	Cytotoxic necrotizing factor 2	Blanco et al., 1996 [34]
*tra*T	GGT GTG GTG CGA TGA GCA CAG CAC GGT TCA GCC ATC CCT GAG	290	Serum resistance	Johnson and Stell, 2000 [8]
*iro*N	AAT CCG GCA AAG AGA CGA ACC GCC T GTT CGG GCA ACC CCT GCT TTG ACT TT	553	Salmochelin siderophore receptor	Johnson et al., 2008 [9]
*omp*T	TCA TCC CGG AAG CCT CCC TCA CTA CTA T TAG CGT TTG CTG CAC TGG CTT CTG ATA C	496	Episomal outer membrane protease	Johnson et al., 2008 [9]
*hly*F	GGC CAC AGT CGT TTA GGG TGC TTA CC GGC GGT TTA GGC ATT CCG ATA CTC AG	450	Putative avian hemolysin	Johnson et al., 2008 [9]
Iss	CAG CAA CCC GAA CCA CTT GAT G AGC ATT GCC AGA GCG GCA GAA	323	Episomal increased serum survival	Johnson et al., 2008 [9]

Table 2: Plasmid and resistance profile of strains from hemocultures.

Strains	Plasmids	Molecular size of the plasmids (MDa)	Resistance profile
1	p1a	78	ApCbCfCmKnTr
2	p2a, b, c, d	113, 112, 86, 82	ApCbSmFoKnTr
3	p3a, b, c, d, e	112, 99, 78, 58, 29	ApCbCfCmSmFoGnKnSiSuTbTcTr
4	p4a	59	ApCfFoTcTr
5	p5a, b	99, 71	ApCbCfCmSmFoKnSuTcTr
6	p6a	112	ApCbCfCmSmFoGnKnSiTcTr
7	p7a	99	ApCbSmFoSu
8	NP	—	ApFo
9	NP	—	ApFo
10	p10a	82	ApCmFoTc
11	p11a	68	ApCbCmSmFo
12	NP	—	Ap
13	p13a, b, c, d, e	87, 68, 59, 50, 29	ApCbCfSmFoGnKnSuTbTcTr
14	p14a, b	62, 44	ApCbSm
15	NP	—	Ap
16	NP	—	Ap
17	p17a	87	ApCfCmSmKnTcTr
18	p18a	62	Ap
19	p19a	92	ApCbCmSmKnSuTcTr
20	NP	—	Ap

NP: no plasmid; Ap: ampicillin; Cb: carbenicillin; Cf: cephalothin; Cm: chloramphenicol; Sm: streptomycin; Fo: fosfomycin; Gn: gentamicin; Kn: kanamycin; Si: sisomicin; Su: sulfonamide; Tb: tobramycin; Tc: tetracycline; Tr: trimethoprim.

the virulence of bacteria during bloodstream infection and sepsis [20, 21]. The PAIs I_{536}, II_{536}, I_{CFT073}, $I_{J96,}$ and II_{J96} harbor a copy of hlyABCD system encoding α-hemolysin, and in our study, six strains harbor *hly*A gene and three also had these PAIs (Table 3). Commensal strains showed a large prevalence of the *hly*A gene too, with 52.94% of the strains, and did not show a good correlation with the PAIs, since only 3 of 27 isolates that had the *hly*A had the respective PAI. Of the virulence genes encoding adhesins (*pap*C, *pap*G, *sfa*A, *sfa*S, and *afa*), *pap*C and *pap*G were the most prevalent in the strains of hemoculture. These two genes were present in 30% of our strains and were always found together in the isolates, including in commensal strains. These genes are part of the mannose-resistant P-fimbriae operon and have been associated with *E. coli* isolated from bacteremia [22, 23]. The PAIs I_{J96}, $I_{CFT073,}$ and II_{CFT073} harbor genes encoding P-fimbriae. Of the six strains of hemocultures containing the genes *pap*C and *pap*G, three contained corresponding PAIs (Table 3). Meanwhile, all the commensal strains containing the *pap*C and *pap*G genes also have PAI I_{CFT073}, showing a good correlation between them.

Siderophore production is important for bacterial survival in the bloodstream. The aerobactin siderophore system is an important virulence factor that contributes to bacterial growth in host tissues and fluids where iron availability is limited [24, 25]. The aerobactin receptor (IutA) is commonly associated with extraintestinal *E. coli* and those isolated from bacteremia [22, 26]. In the present study, *iut*A was the most commonly found virulence gene, present in 65% of the isolates tested (Table 3), different from commensal which did not show this gene. PAI I_{CFT073} contains aerobactin genes, and 5 of the 13 strains containing the *iut*A gene also contained sequences similar to PAI I_{CFT073}. Genes corresponding to other iron uptake systems were also identified: *iro*N, encoding the salmochelin receptor, was present in 55% of isolates; and *fyu*A, encoding the yersiniabactin receptor [24, 25], was present in 45% of isolates. PAI IV_{536} contains yersiniabactin encoding genes, and 8 of the 9 isolates exhibiting the *fyu*A gene contained PAI IV_{536} related sequences. Thus, there is a good correlation between the presence of this island and the genes encoding yersiniabactin. There was a good correlation between the commensal strains too and of the 24 isolates that contained the *fyu*A gene, 22 isolates had also PAI IV_{536}.

Of the genes that confer serum resistance (*kps*II, K1, *tra*T, and *iss*), *tra*T, which encodes an outer membrane lipoprotein that contributes to serum resistance [27] was detected in 50% of the isolates.

The invasion determinant encoded by the *ibe*10 gene was present in 10% of the isolates, whereas the *omp*T gene, encoding an outer membrane protease, was present in 15% of the isolates.

These results demonstrate that the virulence of septicemic *E. coli* is not summarized by the presence of a single virulence factor, since each step in the infection process can be mediated by different virulence factors [14, 28], but the expression of the combination of virulence factors together with the

Table 3: Study of pathogenicity islands, phylogenetic classification, and virulence genes in 20 ExPEC strains from human hemocultures.

Strains	PC	Pathogenicity islands				Virulence genes															
		IV_{536}	I_{CFT073}	II_{CFT073}	I_{J96}	*kps*II	K1	*fyu*A	*pap*C	*pap*G	*sfa*A	*Ibe*10	*hly*	*cnf*1	*cnf*2	*tra*T	*iut*A	*iro*N	*omp*T	*hly*F	*iss*
1	B2	+	+	+	−	+	−	−	+	+	+	−	+	−	−	−	+	+	−	−	−
2	B2	+	+	+	−	+	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−
3	B2	+	+	−	−	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−
4	B2	−	−	−	+	+	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−
5	A	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+
6	A	−	−	−	−	−	−	−	+	+	−	−	−	−	−	+	+	+	−	−	−
7	B2	+	+	−	−	+	−	+	+	+	−	−	+	−	−	+	+	+	−	−	−
8	B2	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
9	B2	+	+	−	−	+	+	+	+	+	+	−	+	−	−	−	+	−	−	−	−
10	B1	+	−	−	−	−	−	+	−	−	−	−	+	−	+	+	−	+	+	+	+
11	A	+	−	−	−	−	−	+	−	−	−	−	+	−	−	+	+	+	−	−	−
12	B1	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
13	A	−	−	−	−	+	+	+	+	+	−	−	+	−	−	+	+	−	−	−	−
14	A	−	−	−	−	+	+	−	+	+	−	−	−	−	−	+	+	+	−	−	−
15	A	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
16	B2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
17	D	+	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	−	−	−	−
18	B1	+	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−
19	D	+	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	+	−	−	+
20	B1	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+

PC: phylogenetic classification; pathogenicity islands: PAI IV_{536}, PAI I_{CFT073}, PAI II_{CFT073}, and PAI I_{J96}; adhesions: *pap*C, *pap*G, *sfa*A, *sfa*S, and *afa*; invasion: *ibe*10; toxins: *cnf*2, *cnf*1, *hly*A, *hly*F, and *cva*C; iron uptake systems: *fyu*A, *iut*A, and *iro*N; serum resistance: *kps* II, K1, *tra*T, and *iss*; proteases: *omp*T.

Table 4: Distribution of pathogenicity islands (PAI) according to phylogenetic classification (PC), among ExPEC and commensal strains.

PC	PAI I_{536}	PAI II_{536}	PAI IV_{536}	PAI I_{J96}	PAI I_{CFT073}	PAI II_{CFT073}	Total of PAIs	Total of strains
B2 (ExPEC)	0	0	6	1	6	2	15	8
B2 (commensal)	0	0	2	0	2	1	5	6
D (ExPEC)	0	0	2	0	0	0	2	2
D (commensal)	0	0	0	0	0	0	0	3
B1 (ExPEC)	0	0	3	0	0	1	3	4
B1 (commensal)	0	0	0	0	2	0	2	5
A (ExPEC)	0	0	1	0	0	0	1	6
A (commensal)	3	3	21	0	7	4	38	37

imbalance between immune defenses and characteristics of the environment determines a multifactorial outcome [29].

Several studies have demonstrated that isolates belonging to phylogenetic group B2 are more commonly extraintestinal pathogenic strains [3, 15, 22]. Our results demonstrated that group B2 (40%) was the most common group among the *E. coli* strains from hemoculture (Table 4). Strains belonging to group B2 also had the most PAI associated sequences, and 15 of the total 22 PAIs identified were present in this group. In contrast, in the commensal strain the most prevalent group was A (72.54%) and this group had a greater number of PAIs (86.95%) (Table 4). Moreover, seven of eight strains of group B2 had PAIs, against only one of six strains of group A. However, despite reports in the literature that isolates belonging to groups A and B1 are more often strictly commensal strains from the intestinal microbiota [3, 7, 15, 22], ten of the isolates sampled belonged to groups A and B1 (Table 3), demonstrating that these groups are also capable of causing systemic infection. The results of the current study indicate that isolates that are phylogenetically characterized as mainly commensal can in some cases be isolated from bloodstream infections, reinforcing the concept that virulence is associated with the presence of multiple virulence factors and is dependent on the host's immune system [29]. The results also showed that group B2 *E. coli*, despite being uncommon among commensal strains, can be present in intestinal flora (11.76% of our commensal strains) (Table 4), suggesting that they may act as a reservoir for bacteria that can cause extraintestinal infection [7].

In previous reports, ExPEC strains from group B2 have been shown to contain more virulence factors than those from groups A and B1 [15, 22, 30]. However, our strains showed on average 4 to 5 virulence factors genes, regardless of the phylogenetic group, and despite the correlation between the presence of virulence genes and strains belonging to phylogenetic group B2, some isolates from group B2 were found to have few virulence genes, whereas some isolates from groups A and B1 had up to 8 virulence genes, while some strains of group B2 with a greater number of virulence factors had 8 virulence genes too (Table 3). Thus, although some isolates from groups A and B1 are limited in their virulence gene content and are not likely to be highly virulent, others in these groups contained multiple virulence factors genes that could contribute to extraintestinal virulence.

Table 5: Genetic characterization of ExPEC transconjugants.

Strains	*iss*	*tra*T	*iut*A	*iro*N	*omp*T	*hly*F
E. coli K12-711	−	−	−	−	−	−
E. coli 5	+	+	−	+	+	+
5.2	+	+	−	+	+	+
E. coli 17	−	+	+	−	−	−
17.1	−	+	+	−	−	−
E. coli 19	+	+	−	−	−	−
19.1	−	+	−	−	−	−

Serum resistance: *iss* and *tra*T; iron uptake systems: *iut*A and *iro*N; proteases: *omp*T; toxin: *hly*F.
Strains (donator of plasmids) = 5, 17, and 19; Tranconjugants = 5.2, 17.1, and 19.1.

Similarly, the virulence genes are not only associated with the PAIs, because some ExPEC also harbor virulence genes in plasmids [31]. Furthermore, PAI are located in regions of high genetic mobility, which show elements that allow recombination and, consequently, PAI rearrangement, deletion, and/or acquisition of foreign DNA [7].

Given the importance of plasmids as mobile elements in the horizontal gene transfer, the plasmid profile was investigated. In this study, 14 strains showed these plasmids, which ranged from 1 to 5 plasmids per strain, and three plasmids transferred to another *E. coli* by conjugation, appearing to be conjugative plasmids. The transconjugants (sample 5, 17, and 19) received genes *iut*A, *omp*T, *hly*F, *iro*N, *tra*T, and *iss*, showing the possible presence of these virulence genes in plasmids (Table 5). Thus, the presence of transferable plasmids in *E. coli* isolated from hemocultures can contribute to the horizontal transfer of virulence genes to nonpathogenic isolates and interestingly the conjugative plasmids 5.2, from strain 5, had *iss*, *iro*N, *omp*T, and *hly*F genes, whose genes are generally present in typical APEC plasmids in a conserved virulence plasmidic (CVP) region [32]. These findings support the idea that APEC or *E. coli* from commercial chicken carcasses has a potential zoonotic risk, as well as serves as a reservoir for virulence genes for ExPEC strains [9, 28, 33].

As ExPEC pathogenicity is due to genetic features such as virulence genes, pathogenicity islands, and plasmids associated with the virulence, the study of genetic factors is important to better understand these important pathogens.

Thus, for the screening and even prevention of blood-borne diseases caused by ExPECs, further research should be conducted on the genetic features associated with the virulence of these pathogens. Although in stool samples of healthy people the presence of commensal strains is common, our results showed that the intestinal microbiota may harbor *E. coli* of phylogenetic group B2. Also *E. coli* with PAIs and virulence genes suggest that the intestinal microbiota may act as a reservoir of ExPEC with virulence genetic factors present at the *E. coli* from blood stream infection.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Improving the Diagnosis of Bloodstream Infections: PCR Coupled with Mass Spectrometry

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The reference method for the diagnosis of bloodstream infections is blood culture followed by biochemical identification and antibiotic susceptibility testing of the isolated pathogen. This process requires 48 to 72 hours. The rapid administration of the most appropriate antimicrobial treatment is crucial for the survival of septic patients; therefore, a rapid method that enables diagnosis directly from analysis of a blood sample without culture is needed. A recently developed platform that couples broad-range PCR amplification of pathogen DNA with electrospray ionization mass spectrometry (PCR/ESI-MS) has the ability to identify virtually any microorganism from direct clinical specimens. To date, two clinical evaluations of the PCR/ESI-MS technology for the diagnosis of bloodstream infections from whole blood have been published. Here we discuss them and describe recent improvements that result in an enhanced sensitivity. Other commercially available assays for the molecular diagnosis of bloodstream infections from whole blood are also reviewed. The use of highly sensitive molecular diagnostic methods in combination with conventional procedures could substantially improve the management of septic patients.

1. Introduction

Bloodstream infection is a life-threatening condition that results from the presence of microorganisms, generally bacteria or fungi, in the blood [1]. The time window for the administration of an appropriate therapy is less than 6 hours once the symptoms are recognized, and it is optimal to administer broad-range antibiotics within the first hour, preferably after obtaining a blood culture for microbiological diagnosis [2]. Inadequate antimicrobial therapy increases the risk of mortality. Every hour of delay in initiation of appropriate antimicrobial therapy increases the mortality by 7.6% in patients with septic shock [3]. Conventional methods for the microbiological diagnosis of sepsis rely on blood culture followed by biochemical identification. It usually takes 1 to 3 days to obtain both the identification and the antimicrobial susceptibility profile of the pathogen. The major limitation of blood culture methods is that they require a median time-to-positivity of 12 to 17 hours [4]. Another limitation of this method is that the presence of unculturable or fastidious microorganisms may decrease its sensitivity. Culture may also be negative if antimicrobial therapy was begun prior to blood sampling. Thus, there is an urgent need to improve the diagnostic tools for a better management of septic patients.

The ideal diagnostic platform should identify a broad spectrum of pathogens (bacteria, fungi, viruses, and protozoa), determine the susceptibility to a battery of antibiotics, allow the analysis of specimens in high or low throughput, have a low cost per sample, have minimum hands-on time, be user friendly, and, ideally, generate the results in a timely manner for the management of septic patients (6 hours or less). Mass spectrometry technology has recently been introduced in the clinical microbiology laboratory. Using matrix-assisted laser ionization time-of-flight (MALDI-TOF)

spectrometers the diagnostic process may be shortened [5–7] as the identification of the pathogen can be achieved within 30 minutes directly from a positive blood culture [8]. Additionally, this technology is able to detect the resistance to some antibiotics [9], such as the presence of β-lactamases (including carbapenemases), methicillin-resistant *Staphylococcus aureus,* and even vancomycin-resistant *Enterococcus* spp. However, this technology relies on culture and, therefore, a median of 12- to 17-hour delay is unavoidable [4]. In order to further accelerate the diagnostic process, it is desirable to detect and identity pathogens directly from the patient's blood, avoiding the culture step.

Several molecular methods have been developed for the detection of pathogens (mainly bacteria and some fungi) in whole blood. The first assays developed were designed for the detection of a single pathogen of interest and are reviewed by Klouche and Schröder [10]. A single-pathogen approach is not useful for the diagnosis of bloodstream infections, as these infections may be caused by a broad range of microorganisms. This limitation has been overcome in several commercial assays which are able to detect a number of microorganisms [10–12]. These assays are based on two main strategies: the identification of a selected group of pathogens using specific targets (i.e., SeptiFast [13], VYOO [14], and Magicplex [15]) or the detection of a broad range of pathogens using universal/conserved targets (i.e., SepsiTest [16], PCR/ESI-MS [17]).

Use of whole blood in assays designed to detect pathogen nucleic acid is challenging. An excess of human DNA may hamper the detection of pathogen genomic material or may inhibit the PCR reaction [18, 19]; hemoglobin traces may also inhibit PCR-based amplification. Therefore, molecular methods are forced to use a relatively small volume of blood (1 to 5 mL); whereas conventional culture methods use 20–30 mL. This limited volume reduces sensitivity of the molecular methods. Additionally, the bacterial load in adults with bloodstream infection can be as low as 1–10 CFU/mL [20], which may preclude detection of pathogen DNA.

The PCR/ESI-MS technology combines broad-range PCR amplification with the electrospray-ionization time-of-flight mass spectrometry, which is a highly sensitive detection method. Methods have now been developed that allow use of the PCR/ESI-MS technology on whole blood samples, and two clinical evaluations of this system have recently been published [21, 22]. A new version of the instrument is presented that has been designed to improve the sensitivity and implementation in the clinical laboratory. This review describes the current status of the molecular diagnosis of sepsis with emphasis on the PCR/ESI-MS technology.

2. Summary of Commercially Available Molecular Assays for the Diagnosis of Bloodstream Infections from Whole Blood

2.1. SeptiFast (Roche, Mannheim, Germany). SeptiFast is a multiplex real-time PCR assay that detects 25 pathogens including five *Candida* species and *Aspergillus fumigatus* [13]. The presence of the resistance gene *mec*A may be detected with a separate test. The initial volume of blood required is 3 mL (using the manual DNA extraction protocol 1.5 mL aliquots are processed in duplicate) or 1.5 mL (using the automated DNA extraction) [23]. The region amplified in this assay is the internal transcribed spacer region (IST), which is located between the 16S and 23S ribosomal genes for bacteria and between 18S and 5.8S ribosomal genes for fungi [13]. The amplification is performed with a LightCycler 2.0 instrument; different pathogens are detected through specific fluorescent probes. The time-to-result using this approach is 4.5–6 hours. This assay has been widely evaluated in the clinical setting; however, the results are conflicting with reported sensitivities ranging from 15% to 98% in ICU patients [24]. Recently, Chang et al. reviewed all the available literature reporting use of the SeptiFast assay and performed a meta-analysis that included data on 6,012 patients from 35 selected studies. The overall calculated sensitivity of SeptiFast was 75.0% (95% confidence interval, 65.0–83.0%), and the specificity was 92.0% (95% confidence interval, 90.0–95.0%). The performance of the test clearly varies depending on the group of patients tested.

2.2. SepsiTest (Molzym, Bremen, Germany). The SepsiTest assay is based on broad-range PCR amplification followed by sequencing. In the SepsiTest two 1 mL aliquots of blood are processed in duplicate and human DNA is selectively degraded prior to the bacterial cell lysis step [16]. Several studies using this approach for the diagnosis of sepsis have been published. The largest study ($N = 342$) [16] reported a sensitivity and specificity of 87.0% and 85.8%. Two smaller studies reported lower values of sensitivity of 46.0% ($N = 50$) [25] and 37.5% ($N = 75$) [26]; specificities were 100% [25] and 86.6% [26].

2.3. VYOO (SIRS-Lab, Jena, Germany). The VYOO assay is a multiplexed PCR analysis that detects 34 pathogens, including six species of *Candida* and *Aspergillus fumigatus,* as well as several resistance genes (methicillin resistance gene *mec*A, vancomycin resistance genes *van*A and *van*B, and β-lactamase genes *bla*SHV and *bla*CTX-M). The amplified products are visualized using a conventional gel electrophoresis, and the time-to-result is 8 hours. For this assay, microbial DNA from 5 mL of blood is enriched: total DNA is applied to an affinity chromatographic column that specifically binds the microbial DNA (LOOXTER) [27]. Additionally, human DNA is depleted during the extraction step. This assay has a sensitivity ranging from 38.0% to 60.0% [14, 25, 28].

2.4. Magicplex Sepsis Real-Time Test (Seegene, Seoul, Korea). In the Magicplex Sepsis assay, three PCR reactions are necessary to achieve the identification at the species level of the pathogen. First, a conventional PCR amplification step is performed. In this step, primers designed to amplify genomic material from 91 microorganisms (85 bacteria, five species of *Candida,* and *Aspergillus fumigatus*) and three resistance genes (methicillin resistance gene *mec*A and vancomycin resistance genes *van*A and *van*B) are used. A real-time PCR is then carried out in a screening step for identification of the

group or genera level of pathogens present. Finally, a second real-time PCR is performed to achieve the identification at species level. Identification of 21 bacterial species, five *Candida* species, and *Aspergillus fumigatus* is possible. For the DNA extraction, 1 mL of whole blood is used and human DNA is removed prior to the lysis of microorganisms. The time-to-result of this assay is 6 hours. To our knowledge, only one study using this approach for the molecular diagnosis of sepsis has been published [15]. The sensitivity and specificity were reported to be 65.0% and 92.0%, respectively.

3. The PCR/ESI-MS Technology

3.1. Principles of the Technology. This technology combines broad-range PCR with ESI-MS mass spectrometry. Briefly, after the PCR, amplicons are desalted and analyzed by mass spectrometry. ESI-MS is used to determine the molecular mass of each amplicon, which is then used to calculate the base composition of each amplicon. The base compositions of multiple amplicons from different regions of the genome are compared to an extensive database and the identification of the pathogen is achieved (Figure 1). Even though the base composition analysis is not as informative as sequencing, it has enough discrimination power for the detection and identification of hundreds of microbial pathogens. A broad bacteria and *Candida* detection assay (BAC assay; Ibis Biosciences, an Abbott company, Carlsbad, CA, USA) has been designed for use in clinical research to identify more than 600 bacteria and *Candida* species. The BAC assay also detects resistance genes for three clinically relevant antibiotics: methicillin (*mec*A), vancomycin (*van*A and *van*B), and carbapenem (*blaKPC*).

3.2. PCR Amplification. The amplification of conserved regions of the genome has been widely used for the identification of microorganisms at the species level. Although the most common targets are the ribosomal DNA genes (i.e., 16S for bacteria and 18S for fungi), several housekeeping genes (i.e., *tufB*, *rplB*, *valsS*, and *rpoB*) are also useful for the identification of pathogens [10, 11, 29]. Within these genes, highly conserved regions are used as priming sites, but the region amplified contains enough variability for the discrimination between species. For instance, in order to identify bacterial and *Candida* species, the BAC assay includes thirteen pairs of primers targeting different conserved regions (nine primers pairs for bacteria and four for *Candida* species). An advantage of using PCR primers designed for several conserved regions with varying degrees of specificity is that when more than one microorganism is present, there is redundancy of coverage across various primer pairs. This is especially relevant when the different microorganisms are present in different abundances, as using several nonoverlapping primer pairs may allow amplification of the less abundant species. Redundant amplification also prevents missed detections due to mismatches in single priming sites [29, 30].

3.3. Detection and Quantification of PCR Products. Mass spectrometry is highly sensitive and can detect small amounts of a nucleic acid of a given sequence even in a complex mixture. The PCR/ESI-MS system employs a software algorithm that calculates a base composition for each amplicon based on mass, compares these to an extensive database, and achieves the identification of the pathogen [17, 31].

Another feature of this technology is that it allows a relative quantification of the microorganism present in the specimen. This is achieved by the use of an internal standard that is amplified with the same primer pairs as those for amplification of the target gene. The internal standard has a different base composition and thus can be differentiated. As this synthetic standard is added to each PCR well at a known copy number, the comparison between standard and microbial DNA permits quantification. In the absence of a PCR product, the internal standard serves as PCR positive control to exclude PCR inhibition.

3.4. Usefulness of the PCR/ESI-MS for the Diagnosis of Bloodstream Infections. The accuracy of BAC assay for the diagnosis of bloodstream infections was first evaluated on blood culture specimens [32–34]. Those studies demonstrated robustness of the technology in terms of accuracy of the identifications. However, with the introduction of MALDI-TOF instruments for the identification of pathogens from positive blood culture based on their protein/peptide profile, it became clear that PCR/ESI-MS would not be able to compete on either a time-to-result or cost-per-sample basis with MALDI-TOF [35].

An advantage of the PCR/ESI-MS assay relative to the MALDI-TOF assay is that PCR/ESI-MS has been optimized to achieve a rapid diagnosis from direct clinical specimens. To date, two clinical evaluations of the PCR/ESI-MS for the diagnosis of bloodstream infections from whole blood have been published. Jordana-Lluch et al. [21] evaluated this system analyzing 247 whole blood specimens (75 with a paired positive blood culture and 172 with a negative blood culture result), and Laffler et al. [22] tested 464 whole blood specimens with a positive paired blood culture and 442 with a negative blood culture result. The agreement between blood culture followed by biochemical identification and PCR/ESI-MS was good in both studies: 77.1% in the Jordana-Lluch et al. study [21] and 78.6% in the Laffler et al. study [22].

Polymicrobial infections were detected in both studies by conventional and/or molecular methods. The agreement between methods on these specimens was low, as most of the mixed infections were detected by only one of the two methods. However, the use of this molecular method in addition to blood culture would have resulted in additional detections of clinically relevant microorganisms in some cases, which could have influenced patient outcome.

In a number of cases in both studies, PCR/ESI-MS detected microorganisms in whole blood specimens with a paired negative blood culture. The clinical relevance of the additionally detected microorganisms was investigated through clinical records review in order to discriminate between probable contaminants and true pathogens. The proportions of detected microorganisms with clinical significance not isolated by conventional methods were 7.5% (13 out

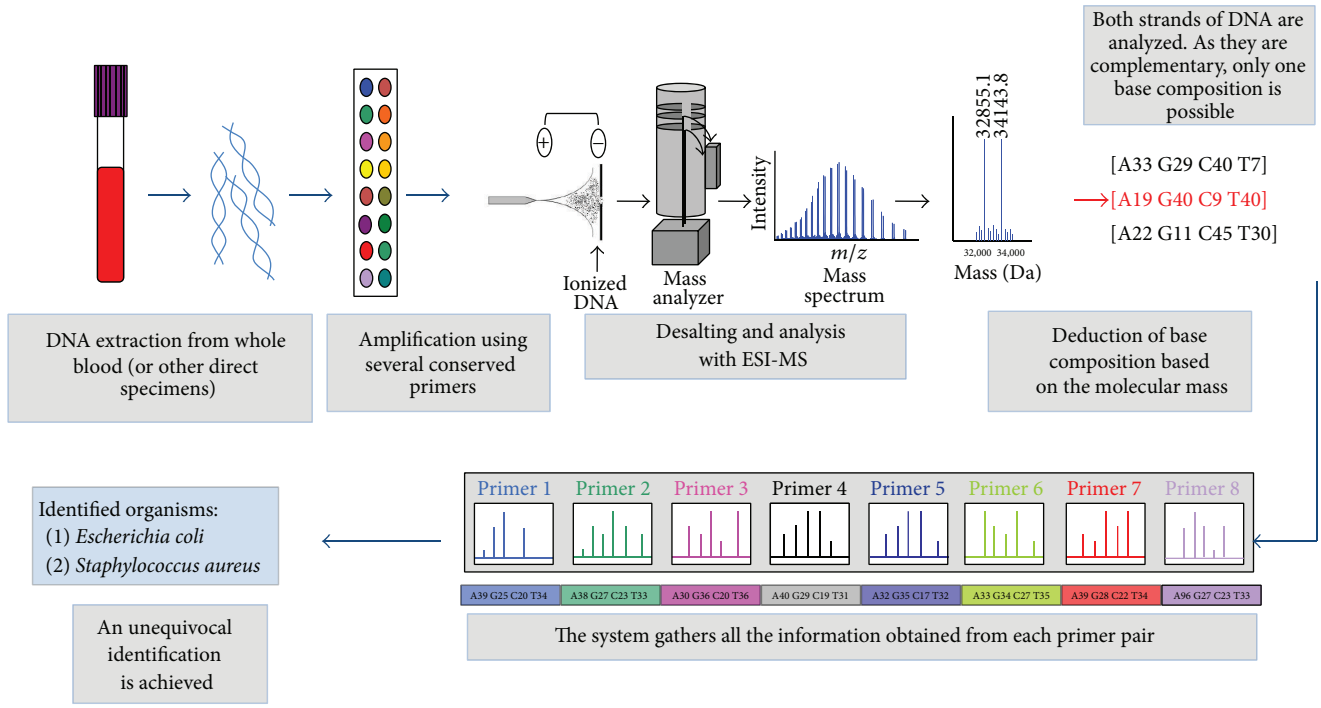

Figure 1: Schematic workflow of PCR/ESI-MS system. ESI-MS: electrospray ionization mass spectrometry. Part of the figure has been modified from Ibis Biosciences, a subsidiary of Abbott Molecular, with permission.

172 blood culture negative cases) [21] and 7.2% (31 out of 431 blood culture negative cases) [22]. These findings are highly relevant, as conventional methods were not able to diagnose the etiology of infection in the culture-negative patients.

The sensitivity of the system was calculated using different approaches in each study. Jordana-Lluch et al. disregarded those specimens with a polymicrobial identification by either or both methods, as the events with one correct detection but with a disagreement in the second one were difficult to catalogue as "true positive" or "false positive." In those terms, the sensitivity of the PCR/ESI-MS was 50.0%. Laffler et al. performed a theoretical approximation of the sensitivity based on the historical blood culture positivity rate in their center. They extrapolated the experimentally obtained PCR/ESI-MS positivity rate in order to obtain the number of negative blood cultures that, if processed by the PCR/ESI-MS, would have additionally tested positive. The estimated sensitivity of PCR/ESI-MS using this theoretical approach was 85.9%. This extrapolation may have led to a biased estimation of the sensitivity.

Although these sensitivity values are not directly comparable because they were calculated in different ways, the Laffler et al. study had a higher detection rate of the PCR/ESI-MS on whole blood specimens with a paired positive blood culture. As many factors may affect the sensitivity of molecular methods, a direct comparison between studies is difficult. Differences in the clinical condition of the patients, their characteristics (e.g., age, antimicrobial treatment at the time of the blood draw), the microorganisms isolated, the number of blood cultures taken, and the volume of blood drawn for culture may result in differences between studies [36]. The limitations in sensitivity of the evaluated version of the PCR/ESI-MS technology result from the amount of blood tested in comparison with the blood culture (1.25 versus 20–30 mL). This problem has been overcome with the new version of the PCR/ESI-MS technology, which uses higher volumes of whole blood reducing the limit of detection 4-5-fold.

3.5. The New Version of PCR/ESI-MS. Since its original description by the team of Ibis Biosciences, the PCR/ESI-MS technology has been continuously evolving. The first instrument, named TIGER (for Triangulation Identification for the Genetic Evaluation of Risk) [31], was initially designed for biodefense and surveillance applications, due to its capability to identify previously unknown and unculturable microorganisms. Shortly after, a commercial version of this technology appeared, the Ibis T5000 [17, 30]. In this format, the sample processing was automated and a software system permitted management of the instrumentation, signal analysis, and report generation. This version of the instrument was intended to be used in health and industry settings; it provided highly sensitive detection without the need for a highly trained operator. With the incorporation of Ibis Biosciences into the Abbott group, the system was upgraded [29]. This system, the PLEX-ID, was used in the aforementioned studies [21, 22, 32, 33, 35]. Recently, a newer version has been developed with improvements focused on the analysis of direct patient specimens. One of the principal changes is the use of a larger volume of blood (5 mL) in order to increase sensitivity. Changes in the extraction process allow

Table 1: Comparison between PLEX-ID and the new version of PCR/ESI-MS.

	PLEX-ID	New version
Volume of whole blood analyzed	1.25 mL	5 mL
Samples per run of nucleic acid extraction	1–24 (24-well plate format, manual dispensation of reagents and specimens)	1–6 (ready-to-use individual reagent cartridges)
Minimum number of samples during MS analysis	6 (96-well plate)	1 (one individual 16-well strip per specimen)
Preanalytical analysis equipment	4 (mechanical lysis, magnetic nucleic acid extraction, fluid handler, and thermocycler)	3 (mechanical lysis, magnetic nucleic acid extraction, and thermocycler)
Analytic equipment	1 large instrument (desalting and MS in the same instrument)	2 bench-top instruments (separation of desalting and MS)
Time-to-result	6 h	5-6 h

ESI-MS: electrospray ionization mass spectrometry.

the use of several types of primary tubes and extraction protocols are tailored to the needs of the clinical laboratory. Another important improvement is that one to six specimens can be analyzed at a time. Finally, the mass spectrometer is a bench-top instrument, facilitating installation in clinical laboratories. In Table 1, a comparison between the PLEX-ID and the new version of the PCR/ESI-MS technology is depicted. A preliminary evaluation of this new version has shown a better sensitivity in the detection of pathogens in direct clinical specimens. Further evaluations are currently underway.

3.6. Other Applications in the Clinical Diagnosis of Infectious Diseases. The versatility of the PCR/ESI-MS has been widely demonstrated. In 2012, Wolk et al. [37] reviewed the existing literature of this technology. In this section, we aim to summarize its potential applications in the clinical laboratory as well as to point out several new publications not included in the previous review.

A PCR/ESI-MS assay is able to differentiate species in the *Mycobacterium tuberculosis* complex and classify these species based on drug resistance [38, 39]. This technology has also proved its usefulness for epidemiological proposes, given that it enables molecular genotyping [40]. For instance, genotyping of *Staphylococcus aureus* [41, 42], *Acinetobacter baumannii* [43–45], and respiratory pathogens [46, 47] has been performed in a variety of clinical settings. Bhatia et al. [48] used PCR/ESI-MS to identify a *Streptococcus intermedius* species from cerebrospinal fluid (CFS) and from a fixed biopsy in a patient with a central nervous system (CNS) infection. Although this infection had a respiratory origin, both bronchoalveolar lavage and CFS cultures were negative. Farrell et al. [49] investigated the capability of PCR/ESI-MS to identify pathogens on several specimens collected from patients undergoing antimicrobial treatment. A total of 76 clinical specimens including swabs, blood cultures, fluids, and tissues were collected from 47 patients. From those, 72% (55/76) were culture negative, whereas 76% (58/76) were PCR/ESI-MS positive.

Major viral families can also be detected using this approach. Of special interest is the new version of the Viral IC assay designed for the diagnosis of opportunistic viral infections of immunocompromised patients by viruses such as *Herpesvirus*, *Adenovirus*, *Parvovirus*, *Picornavirus*, and *Polyomavirus*. The ability of the assays on the PCR/ESI-MS system to detect influenza virus, coronavirus, respiratory syncytial virus, human adenovirus, human metapneumovirus, vector-borne flaviviruses, and alphaviruses has been demonstrated [50–52]. Moreover, this technology shows a great promise for the global surveillance of influenza virus [53–55]. Remarkably, it was able to detect the novel H1N1 strain during the 2009 influenza virus outbreak without any modification in the Influenza Surveillance Assay (Ibis Biosciences, Carlsbad, CA, USA) [56].

Fungi are causative agents of infections, but due to the slow growth of these microorganisms, identification by culture is often impractical. Recently, a new assay for the PCR/ESI-MS systems has been validated for detection of *Aspergillus* spp., *Candida* spp., *Pneumocystis* spp., *Cryptococcus* spp., *Mucor* spp., and *Rhizopus* spp. [57]. Concordance rates between PCR/ESI-MS and phenotypic identification and sequencing were 89.7% at the genus level and 87.4% at the species level. Although most of the experiments in this study were performed with reference strains and clinical isolates, detection of *Aspergillus terreus* directly from a culture-negative bronchioalveolar lavage was demonstrated [58].

4. Conclusions

Microbiological diagnosis has historically relied on culture. Isolation of the causal agent provides an irrefutable proof of an infection and allows pathogen identification and determination of antibiotic susceptibility. However, many microorganisms are unculturable, fastidious, or slow-growing. Additionally, prior antimicrobial treatment negatively affects culture-based tests. In the case of bloodstream infections, lack of detection is critical. A significant percentage of blood cultures are negative despite the high likelihood of a bacterial or fungal infection [2]. Lack of culturability and the time to answer mean that many septic patients are not appropriately treated. PCR/ESI-MS is a robust technology that offers a rapid alternative for the diagnosis of bloodstream as well as

other infections. Although being not currently commercially available, the new presentation of the technology has been improved in several aspects that significantly enhance sensitivity. The main advantage of this technology is that it can be used on direct patient specimens, avoiding the culture step. Using this technology as a complement to conventional methods will offer a real improvement in the management of septic and other critically ill patients (i.e., patients suffering from meningitis or fever of unknown origin). Its versatility for the detection of different kinds of microorganisms will make this technology a highly valuable tool in the clinical laboratory.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Bacteriological Profile and Drug Resistance Patterns of Blood Culture Isolates in a Tertiary Care Nephrourology Teaching Institute

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Blood stream infections can lead to life threatening sepsis and require rapid antimicrobial treatment. The organisms implicated in these infections vary with the geographical alteration. Infections caused by MDR organisms are more likely to increase the risk of death in these patients. The present study was aimed to study the profile of organisms causing bacteremia and understand antibiotic resistance patterns in our hospital. 1440 blood samples collected over a year from clinically suspected cases of bacteremia were studied. The isolates were identified by standard biochemical tests and antimicrobial resistance patterns were determined by CLSI guidelines. Positive blood cultures were obtained in 9.2% of cases of which Gram-positive bacteria accounted for 58.3% of cases with staph aureus predominance; gram negative bacteria accounted for 40.2% with enterobactereciea predominence; and 1.5% were fungal isolates. The most sensitive drugs for Gram-positive isolates were vancomycin, teicoplanin, daptomycin, linezolid, and tigecycline and for Gram-negative were carbapenems, colistin, aminoglycosides, and tigecycline. The prevalence of MRSA and vancomycin resistance was 70.6% and 21.6%, respectively. ESBL prevalence was 39.6%. Overall low positive rates of blood culture were observed.

1. Introduction

Blood stream infections range from self-limiting infections to life threatening sepsis that requires rapid and aggressive antimicrobial treatment [1]. A wide spectrum of organisms has been described that cause blood stream infections and this spectrum is subject to geographical alteration [2–5]. Increasing antimicrobial resistance is a worldwide concern. The prevalence of resistance of blood borne isolates is increasing and it also varies in accordance with geographical and regional location. The infection caused by MDR organisms is more likely to prolong the hospital stay, increase the risk of death, and require treatment with more expensive antibiotics. In almost all cases, antimicrobial therapy is initiated empirically before the results of blood culture are available. Keeping in mind the high mortality and morbidity associated with septicemia, right choice of empiric therapy is of importance [6]. Therefore, the present study was undertaken to analyze the various organisms causing septicemia and their antibiotic resistance patterns, as it would be a useful guide for clinicians initiating the empiric antibiotic therapy.

2. Materials and Methods

A total of 1440 samples from clinically suspected cases of bacteremia were studied at Muljibhai Patel Urological Hospital for a period of one year from October 2012 to September 2013. Our institute is 140-bedded teaching hospital which caters to all kinds of nephrology and urology patients including moderate size of hemodialysis programme as well as kidney transplant programme. All the samples were collected from indoor patients in our hospital during the study period and processed in the central laboratory.

Blood was collected from 2 different sites (avg. 8 mL per site) 20 minutes apart in every patient using strict aseptic

precautions and inoculated immediately into BacT/ALERT FA plus—aerobic blood culture bottles with 0.025% of sodium polyanethol sulfonate as anticoagulant. In pediatric cases 1-2 mL of blood was inoculated in BacT/ALERT PF plus pediatric blood culture bottles. After collection these bottles were immediately incubated in BacT/ALERT 3D (manufactured by bioMerieoux)—a fully automated blood culture system for detection of growth in blood culture. The negative results were followed up to 7 days and final report was issued. While, in case of a positive growth, the BacT/ALERT automatically gives an alert. The positive bottles were then subcultured on chrome agar. From the colonies on chrome agar 0.5 McFarland suspension was prepared which was then subjected to identification and susceptibility testing on Mini API ($n = 50$) till February 2013 or Vitek 2 ($n = 82$) from March 2013 onwards (manufactured by bioMerieoux)—which is a fully automated system for identification of organism and antimicrobial susceptibility testing as per the CLSI 2013 guidelines. The ESBL status was determined by Mini API/Vitek 2 as per the CLSI guidelines and was not subjected to any further testing.

3. Results

During the study period, 1440 blood cultures were analyzed of which 132 microorganisms were isolated, out of which 130 were bacterial isolates and 2 were fungal isolate, that is, *Candida albicans*. Their mean age was 48.6 ± 14.8 years of which 89 were males and 43 were females. During study period we did not observe multiple positive blood cultures from any patient. The distribution and percentage of various bacterial and fungal isolates are shown in Table 1.

Among the Gram-positive isolates, the predominant isolate was *Staphylococcus aureus* as shown in Table 2 which exhibited least resistance to tetracycline, doxycycline, vancomycin, teicoplanin, tigecycline, daptomycin, and linezolid. Oxacillin resistance (MRSA) was 70.6% in these strains. Vancomycin resistance in *Staphylococcus aureus* isolates was 21.6%. The vancomycin intermediate *Staphylococcus aureus* was observed in 1 patient. In VRSA strains the MIC for vancomycin was ≥32. This was not confirmed further by reference MIC testing.

Other Gram-positive isolates coagulase negative staphylococcal strains (CONS) showed least resistance to gentamicin, quinolones, co-trimoxazole, tigecycline, linezolid, and tetracycline. Kocuria rosea showed no resistance to tetracycline and co-trimoxazole while *Micrococcus* showed least resistance to tetracycline, vancomycin, tigecycline, and levofloxacin. Enterococci showed least resistance to tetracycline, teicoplanin, and tigecycline. *Streptococcus* was isolated in one case only.

Among the Gram-negative isolates, the predominant isolates were *E. coli* and *Klebsiella* in 33 of 53 (62.3%) as highlighted in Table 3 of which 21 (39.6%) were ESBL producers. *E. coli* isolates showed least resistance to carbapenems, aminoglycosides, and tigecycline and moderate resistance to beta-lactam beta-lactamase inhibitors. *Klebsiella* showed least resistance to carbapenems and moderate resistance to aminoglycosides, tigecycline, and beta-lactam beta-lactamase inhibitor combination. *Pseudomonas* showed least resistance to carbapenems, piperacillin-tazobactam, and aminoglycosides. Other Gram-negative isolates were *Burkholderia* in 6 cultures, *Sphingomonas* in 4, *Acinetobacter* in 2, and *Moraxella* in 1 culture. Eight isolates including 2 CONS, 2 *Micrococcus*, 2 *Sphingomonas*, *1 Burkholderia*, and 1 *Moraxella* were considered contaminated based on clinical and supporting laboratory indicators.

Table 1: Distribution of isolates in blood cultures.

Type	Numbers	Percentage
Staphylococcus	51	38.6
E. coli	20	15.2
Klebsiella	13	9.8
Pseudomonas	7	5.3
Kocuria	7	5.3
Micrococcus	7	5.3
Burkholderia	6	4.5
Coagulase-negative staphylococci	6	4.5
Enterococcus	5	3.8
Sphingomonas	4	3.0
Candida	2	1.5
Acinetobacter	2	1.5
Moraxella	1	0.8
Streptococcus	1	0.8

4. Discussion

In the present paper, blood culture positivity was seen in 132 of 1440 (9.2%) cases which is quite similar to Mehta et al. [7] and China and Gupta [8] but quite lower to other studies of Kamga et al. [9], Kavitha et al. [10], and Roy et al. [11]. We feel the low incidence in our paper is due to various reasons. Majority of the patients reported to us are referred by other specialists or hospitals and these patients were offered antibiotics elsewhere before they reached our hospital. Many patients developed infections after hospitalization or after surgery by which they already had been given antibiotics before sampling of blood for culture.

The incidence of Gram-positive organisms was 77/132 (58.3%) while 53/132 (40.2%) were Gram-negative isolates in our paper. It is in accordance with the studies of China and Gupta [8], Kamga et al. [9], Anbumani et al. [12], and Karlowsky et al. [13] who reported similar incidencesbut in most of the studies like Mehta et al. [7], Mehdinejad et al. [14], Barati et al. [15], and Ayobola et al. [16] Gram-negative organisms have taken over Gram-positive organisms in hospital settings. This difference could be related to an active dialysis programme and substantial contribution of dialysis line or catheter related infections which are usually of Gram-positive nature. This also indicates that infections by Gram-positive organisms constitute a significant threat to septicemia in our locale and the spectrum of organisms is subject to geographical alterations.

Table 2: Drug resistance pattern of major Gram-positive isolates.

Antimicrobial tested	*Enterococcus* ($n = 5$)		*Staphylococcus aureus* ($n = 51$)	
	Mini API ($n = 3$)	Vitek ($n = 2$)	Mini API ($n = 21$)	Vitek ($n = 30$)
Amoxicillin	NP	NP	100	100
Cefotaxime + clavulanate	NP	50	NP	37
Ceftazidime + clavulanate	NP	50	NP	37
Cefepime + tazobactam	NP	50	NP	30
Tigecycline	NP	0	NP	NP
Gentamycin	33	50	NP	NP
Amikacin	33	50	NP	NP
Ciproflox	100	100	90	87
Levoflox	100	100	75	80
Nalidixic acid	100	100	NP	NP
Nitrofurantoin	100	100	0	78
Tetracycline	33	50	10	27
Doxycycline	33	50	10	27
Minocycline	33	50	10	27
Oxacillin	NP	NP	73	67
Vancomycin**	50	50	20	22
Teicoplanin	33	50	NP	0
Daptomycin	NP	0	NP	6
Linezolid	NP	0	NP	3

NP: drug not in panel; ** see Section 4.
Figures in the table are expressed in percentages.

Table 3: Drug resistance pattern of major Gram-negative isolates.

Antimicrobial tested	*E. Coli* ($n = 20$)		*Klebsiella* ($n = 13$)##	*Pseudomonas* ($n = 7$)	
	Mini API ($n = 12$)	Vitek ($n = 8$)	Vitek ($n = 13$)	Mini API ($n = 1$)	Vitek ($n = 6$)
Amoxicillin	92	100	100	100	100
Ceftriaxone	92	100	100	100	100
Ceftazidime	92	87.50	92	0	67
Cefepime	84	75	92	0	50
Amoxicillin + clavulanate	84	100	100	100	100
Cefotaxime + clavulanate	40	37.50	72	100	83
Ceftazidime + clavulanate	40	37.50	54	100	67
Ticarcillin + clavulanate	58	NP	NP	0	NP
Piperacillin + tazobactam	50	50	77	0	33
Cefoperazone + sulbactam	NP	12.50	62	NP	33
Cefepime + tazobactam	NP	37.50	54	NP	33
Imipenem	10	0	54	0	33
Meropenem	10	12.50	70	0	33
Ertapenem	NP	NP	62	NP	NP
Colistin	NP	0	8	0	0
Tigecycline	NP	0	54	NP	67
Gentamycin	42	37.50	54	0	33
Amikacin	30	12.50	46	0	33
Ciproflox	92	87.50	77	0	33
Levoflox	92	87.50	77	0	33
Nalidixic acid	92	87.50	84	NP	NP
Nitrofurantoin	58	70	84	100	83
Tetracycline	75	75	84	100	83
Doxycycline	75	75	84	100	83
Minocycline	75	75	84	100	83

Figures are in percentage; N.P: drug not in panel; ## all the *Klebsiella* isolates were tested on Vitek 2.

Staphylococcus was isolated in 38.6% ($n = 51$) of cases and CONS in 4.5% of cases in the present paper. The isolation of *Staphylococcus aureus* is consistent with the study of Arora and Devi [17], Roy et al. [11], and Karlowsky et al. [13] where the reported isolation of the organism was 27.3%, 14%, and 16.5%, respectively. However, reported isolation of CONS was 20.16%, 16.5%, and 42%, respectively, in these studies which is quite higher than isolation of CONS seen in our study but in accordance with Anbumani et al. [12] where *Staphylococcus aureus* is reported as 36.4% and CONS as 1.12%. Given that CONS isolated from blood are often skin contaminants which are clinically insignificant [1–5], we suspect that the observed low isolation of CONS in our paper could be due to or related to strict aseptic practices of collection method followed for blood sampling of blood culture. The burden of other Gram-positive isolates was much lesser than *Staphylococcus aureus* which is in accordance with these studies.

Enterococcus was isolated in 3.8% ($n = 5$) of cases. Out of these 4 were *Enterococcus faecalis* and 1 was *Enterococcus gallinarum*. Amongst the 4 *Enterococcus faecalis* 2 were vancomycin sensitive and 2 were vancomycin resistant, while *Enterococcus gallinarum* was moderately sensitive to vancomycin.

E. coli and *Klebsiella* (25%) were the predominant Gram-negative isolates in our paper which is in accordance with other studies of Mehta et al. [7], Karlowsky et al. [13], Kamga et al. [9], and China and Gupta [8]. We also observed similar frequency of *Pseudomonas* and *Acinetobacter* as in these studies but we did not observe any isolate of *Salmonella* which is isolated in the frequency of 10 to 20% in these studies. Generally, *Salmonella* is community-acquired infection in general population which gains entry via feco-oral route. Since we cater to specific renal population, that might possibly be the causative factor for this observed difference of *Salmonella*.

We also observed that significant proportion of our patient pool is immunocompromised due to CKD status or postkidney transplantation status which led to bacteremia with various organisms like *Burkholderia*, *Sphingomonas*, *Moraxella*, *Kocuria*, and *Micrococcus* which commonly does not lead to bacteremia in healthy nonimmunocompromised individuals.

Staphylococcus aureus isolates in our study exhibited oxacillin resistance of 70.6% which is quite high from the studies of Kamga et al. [9], Kavitha et al. [10], China and Gupta, [8] and Karlowsky et al. [13] who reported a percentage of 18%, 40.8%, 49.5%, and 29%. This is in accordance with the studies of Garg et al. [6] who reported a percentage of 75.6%. Vancomycin resistance in our *Staphylococcus* isolates was 21.6% which is in accordance with Kamga et al. [9] who recorded an isolation of 32%. But it is in contrast to the studies of Karlowsky et al. [13], China and Gupta [8], Garg et al. [6], Kavitha et al. [10], and Roy et al. [11] who reported no resistance to vancomycin. The increasing glycopeptide resistance in our study could be due to widespread usage of the drug in the empirical treatment protocol of suspected CRBSI in dialysis population. However, the current resistance pattern emphasizes the importance of strict antibiotic policy to prevent emergence and spread of antibiotic resistance.

In view of significant oxacillin and vancomycin resistance of 70.6% and 21.6%, respectively, in staphylococcal isolates, drugs like clindamycin, linezolid, daptomycin, and teicoplanin should be considered in the treatment of MRSA before vancomycin. Vancomycin resistant *Enterococcus* (VRE) in our study is 40% (2/5) which is in accordance with the studies like Garg et al. [6] and Karlowsky et al. [13] who reported 16.6% and 35.8%, respectively.

Among the Gram-negative isolates, the Enterobacteriaceae isolates in our study showed very poor sensitivity to quinolones, penicillins, and cephalosporins. However combining BL+BLI did improve the sensitivity. Least resistance was observed with carbapenems, colistin, aminoglycosides, and tigecycline. We could not compare the sensitivity pattern of carbapenems, colistin, and tigecycline with other studies as these drugs were not tested in majority of the other studies.

ESBL producers detected in our study were 39.6% which is in accordance with the study of Kavitha et al. [10] and Arora and Devi [17] who reported prevalence of ESBL producers as 32% and 34.4%, respectively.

5. Conclusion

Staphylococcus aureus and organisms belonging to Enterobacteriaceae family are the leading causes of septicemia. The most sensitive drugs for Gram-positive isolates were tetracycline, teicoplanin, vancomycin, clindamycin, daptomycin, and linezolid and the most sensitive drugs for Gram-negative bacteria were carbapenems, colistin, aminoglycosides, and tigecycline. Clinicians should exercise caution in their use of vancomycin in order to preserve this useful antibiotic and prolong its therapeutic usefulness and replace its use by drugs like teicoplanin, daptomycin, and linezolid. Increasing incidence of drug resistant organisms like MRSA, VRE, and ESBL producers raises serious concerns about antibiotic resistance and mandates strict antibiotic policy on a large scale. As the practice of prescribing antibiotics is completely unregulated, cheap generics are available, usage of all kinds of antibiotics for even minor illnesses is widespread, and there are not many newer antimicrobials in research pipeline, it is foreseen that if the same kind of practice and scenario continues the antibiotic resistance is likely to go up and we will face serious crisis of antibiotics in near future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Biomarkers for Sepsis

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Bloodstream infections are a major concern because of high levels of antibiotic consumption and of the increasing prevalence of antimicrobial resistance. Bacteraemia is identified in a small percentage of patients with signs and symptoms of sepsis. Biomarkers are widely used in clinical practice and they are useful for monitoring the infectious process. Procalcitonin (PCT) and C-reactive protein (CRP) have been most widely used, but even these have limited abilities to distinguish sepsis from other inflammatory conditions or to predict outcome. PCT has been used to guide empirical antibacterial therapy in patients with respiratory infections and help to determine if antibacterial therapy can be stopped. New biomarkers such as those in this review will discuss the major types of biomarkers of bloodstream infections/sepsis, including soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), soluble urokinase-type plasminogen receptor (suPAR), proadrenomedullin (ProADM), and presepsin.

1. Introduction

"Sepsis is a state caused by microbial invasion from a local infectious source into the bloodstream which leads to signs of systemic illness in remote organs," this was the first scientific definition of sepsis proposed by Dr. Schottmuller in 1914 [1]. Thus, bloodstream infection or bacteremia was a condition to the diagnosis of sepsis and this definition did not change significantly over the years. Sepsis, septicemia, and bloodstream infections (bacteremia) were considered to refer to the same clinical condition, and, in practice, the terms were often used interchangeably. Now, we know that less than one-half of the patients who have signs and symptoms of sepsis have positive blood culture or other microbiological proof of an infectious focus [2].

Bloodstream infections are a major concern to physicians because of high levels of antibiotic consumption and of the increasing prevalence of antimicrobial resistance. Thus, they lack accuracy to tailor subsequent therapy.

Blood cultures to detect bloodstream infections are the mainstay of such attempts when patients do not display localizing signs or symptoms. The presence of SIRS has been shown to increase the likelihood that the blood culture will be positive but blood cultures are often negative in patients with clinical sepsis [3].

Bloodstream infections can produce an immune response to bacterial endotoxins. Innate immune response stimulates macrophages to produce tumor necrosis factor (TNF), interleukin-1β, and interleukin-6. These three proinflammatory cytokines produce a systemic inflammatory response syndrome (SIRS) which is characteristic of early sepsis. A compensatory anti-inflammatory response syndrome (CARS) has been described by Bone [4] that often follows the hyperinflammatory phase, especially in patients who develop what is called "severe" sepsis. In severe sepsis, evidence of widespread organ dysfunction is also present, including multiorgan dysfunction (lung, liver, and/or kidney injury). The so-called septic shock, in which patients suffer cardiovascular collapse unresponsive to fluid resuscitation and vasopressor therapy, is often the terminal event of severe sepsis [5].

However, no gold standard exists for proof of infection. Bacteremia is identified in only about 30% of patients with sepsis, depending on previous antibiotic treatment [6].

Biomarkers can add accuracy of any bacterial presence and they are useful to monitoring the evolution of infectious

process. New biomarkers related to infectious diseases have been tested the last years but few of them, however, have gone through the hurdles of rigorous testing to be used in the clinical practice [7].

Several biomarkers are already available for clinical use in sepsis; however, their effectiveness in many instances is limited by the lack of specificity and sensitivity. Other factors include limitation to characterize the presence of an infection and the complexity of the inflammatory and immune processes to stratify patients into homogenous groups for specific treatments [8].

Many biomarkers can be used in sepsis, but none has sufficient specificity or sensitivity to be routinely employed in clinical practice. PCT and CRP have been most widely used, but even these have limited abilities to distinguish sepsis from other inflammatory conditions or to predict outcome. In view of the complexity of the sepsis response, it is unlikely that a single ideal biomarker will ever be found [9].

In the 1980s, there were numerous studies about the C-reactive protein (CRP), a well-established member of the group of proteins synthesised in the liver. In the 1990s, investigators discovered that the levels of procalcitonin (PCT), the precursor of the hormone calcitonin, were elevated in patients with bacterial infection [10]. Elevations of both CRP and PCT were added to the updated definition of sepsis in 2003. Then, in the early part of the past decade, clinical guides of intensive "goal-directed" treatment of severe sepsis and septic shock used elevated lactate levels to guide therapy, and obtaining a lactate level when monitoring patients at risk of developing sepsis became standard practice [11].

No single biomarker of bloodstream infections may be ideal, but many are helpful in terms of identifying bacterial infections in critically ill patients who need close monitoring so that the antibiotic therapy may be modified or stopped as soon as possible. This review will discuss the major types of biomarkers of bloodstream infections/sepsis which have been tested in different conditions.

2. CRP

CRP is a protein produced in response to infection and/or inflammation and it is widely used in clinical tests to diagnose and manage patients with sepsis. This biomarker is an acute phase reactant whose synthesis in the liver is upregulated by IL-6. The CRP's role during acute inflammation is not entirely clear and it may bind the phospholipid components of microorganisms, facilitating their removal by macrophages. Because the levels of CRP rise significantly during acute inflammation, this biomarker has been used for decades to indicate the presence of significant inflammatory or infectious disease, especially in pediatrics [12]. Although its low specificity may be its primary drawback as a biomarker of sepsis in adults, it is commonly used to screen for early onset sepsis in neonatology [13].

3. Procalcitonin

Procalcitonin is a prohormone (peptide precursor) of calcitonin that is released by parenchymal cells, including liver cells, kidney cells, adipocytes, and muscle cells in response to bacterial toxins, leading to elevated serum levels (up to 5000-fold) within 2 to 4 hours; in contrast, procalcitonin is downregulated in patients with viral infections [14]. The biological half-life of PCT is 22 to 26 hours, an advantageous time point compared with CRP and other acute-phase reactants [15].

Although elevations of PCT can be observed in non-infectious disorders, especially following trauma [16], at present, PCT levels have been used to guide empirical antibacterial therapy in patients with acute exacerbations of chronic bronchitis, community-acquired pneumonia (CAP), and sepsis. Also, PCT levels, along with standard clinical parameters, can assist in determining whether the patient's empirical antibacterial therapy is effective [17]. Higher PCT levels have been associated with increased mortality rates and correlated with severity scores (APACHE, SOFA, and SAPS) [18]. Finally, the most useful application is the use of sequential PCT levels to determine if antibacterial therapy can be stopped [19].

3.1. Procalcitonin for the Guidance of Antibiotic Therapy in Lower Respiratory Tract Infections. Numerous studies have evaluated PCT as a biomarker to guide initiation of antibiotic therapy in patients suspected of lower respiratory tract infections. A meta-analysis published in 2011 with 8 studies (3431 patients) showed a reduction in antibiotic prescription in the PCT-guided antibiotic treatment groups with a RR: 0.69 (CI 95%: 0.55 to 0.88) but with a significant heterogeneity (χ^2 = 192.34; $P < 0.001$, I^2 = 96.9%) [20]. As PCT levels increase upon bacterial infection and decrease upon recovery, it can be used to guide antibiotic therapy in individual patients as a surrogate biomarker. Two low PCT measurements, over the first 4 to 6 hours of hospital admission, resulted in fewer patients started on empirical antibacterials. Low PCT levels over the first 4 hours of inpatient care have an excellent negative predictive value for bacterial infection [21].

A Cochrane review published in 2012 with 14 studies (4221 participants) showed that PCT guidance was not associated with increased mortality (5.7% versus 6.3%, adjusted OR 0.94, 95% CI 0.71 to 1.23) or treatment failure (19.1% versus 21.9%). Total antibiotic exposure was significantly reduced overall [21]. Similar results were founded in a recent meta-analysis including 7 studies (1075 patients) with a hazard ratio of 1.27, 95% CI: 1.01–1.53 reduction of antimicrobial therapy [22].

To date, numerous studies (including meta-analysis) have been published and provide consistent results that withhold antibiotic prescription can be done with low levels of PCT (<0.25 ng/mL) [23].

3.2. Procalcitonin for Antibiotic Guidance in Other Infections. Procalcitonin has been studied in febrile neutropenic patients, fungal infections, postoperative fever, arthritis, endocarditis, meningitis, and suspected bloodstream infections [24–26]. The majority of published studies were observational and it remains uncertain whether PCT can be safely used for antibiotic guidance in different settings. For some infections, PCT may not be sensitive enough for routine

clinical use. In a recent meta-analysis with 6 trials (1006 episodes of suspected endocarditis), the global measures of accuracy of CRP were higher than PCT showing that current evidence does not support the routine use of serum PCT or CRP to rule in or rule out endocarditis [27].

3.3. Procalcitonin for Identification of Sepsis. Procalcitonin has been studied to differentiate between sepsis and systemic inflammatory response syndrome of noninfectious origin. Numerous studies have investigated the diagnostic usefulness of PCT, comparing it with CRP. Initially, PCT was found more sensitive and specific than CRP for bacterial infection [28].

In a meta-analysis of Uzzan et al. (publication date: 2006), 33 studies published between April, 1996, and October, 2004, were included, with 3,943 patients (1,825 patients with sepsis, severe sepsis, or septic shock and 1,545 with only systemic inflammatory response syndrome). This meta-analysis showed that the summary receiver operating characteristics curve for PCT was higher than for CRP for identification of sepsis (0.78 versus 0.71, $P = 0.02$). However, the investigators restricted the population to surgery or trauma patients. Therefore, no conclusion can be drawn for patients other than surgical [29].

A posterior meta-analysis (2007) looking at the diagnostic accuracy of PCT in sepsis diagnosis in critically ill patients included 18 studies published between April, 1996, and November, 2005, with very restrictive inclusion criteria, including evidence of infection by any microbiological test. Uzzan et al. concluded that PCT was not able to discriminate between sepsis and systemic inflammatory response syndrome. The diagnostic accuracy of PCT was low, mean sensitivity and specificity were both 71% (95% CI 67–76), and the area under the summary receiver operator characteristic curve was 0.78 (95% CI 0.73–83). However, their findings were heavily biased because of their selection criteria. The rejection of such studies has been raised as a major criticism of their conclusion that PCT cannot accurately distinguish sepsis from SIRS in critically ill patients [30].

The most recent meta-analysis published by Tang et al. included 30 studies (3244 patients) until February 2012. They concluded that accuracy of PCT to discriminate sepsis and systemic inflammatory response was low, mean sensitivity 77% (95% 72–81), and specificity 79% (95% CI 74–84). The area under the receiver operating characteristic curve was 0.85 (95% CI 0.81–0.88), with substantial heterogeneity (I^2: 96%, 95% CI 94–99) [31].

Although PCT has been shown to correlate closely with infection, it has some limitations. It rises transiently in patients with nonseptic conditions and systemic inflammatory response syndromes (SIRS) (e.g., trauma, surgery, and heatstroke) and is not detectable in certain cases of sepsis [32].

4. New Biomarkers

There are new biomarkers tested for acute infections with different diagnostic and prognostic value (see Table 1). In adults, the soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), soluble urokinase-type plasminogen receptor (suPAR), proadrenomedullin (pro-ADM), and presepsin appear promising because of acceptable sensitivity and specificity [7] (see Table 2).

Table 1: Role of biomarkers of sepsis.

Biomarkers of sepsis	Prognostic value	Diagnostic value	Syndrome/disease
CRP	No	Yes	Sepsis
Procalcitonin	Yes	Yes	Sepsis/respiratory tract infections/pneumonia/
sTREM-1	Yes	Yes	Sepsis/pneumonia/ meningitis
Pro-ADM	Yes	No	Pneumonia
suPAR	Yes	No	Sepsis/tuberculosis
Presepsin	Yes	Yes	SIRS/sepsis

4.1. sTREM-1. The triggering receptor expressed on myeloid cells-1 (TREM-1) is a member of the immunoglobulin superfamily. Its expression on phagocytes is upregulated by exposure to bacteria and fungi. A soluble form of TREM-1 (sTREM-1) can be found in body fluids, such as plasma, pleural fluid, bronchoalveolar lavage fluid, urine, and cerebrospinal fluid, where it can be assayed by ELISA using commercial immunoassay kits [33].

Clinical studies of the ability of the soluble form of TREM-1 to reliably identify patients with sepsis have not been promising [34]. However a meta-analysis of 11 studies (1795 patients included) showed a pooled sensitivity and specificity of 79% (95% confidence interval (CI), 65 to 89) and 80% (95% CI, 69 to 88), respectively with ROC curve of 0.87 (95% CI, 0.84 to 0.89). In this meta-analysis, for a prevalence of 62% of sepsis, the negative predictive value (NPV) was 0.7 and the positive predictive value (PPV) is 0.86. Finally, plasma sTREM-1 had a moderate diagnostic performance in differentiating sepsis from SIRS and was not sufficient for sepsis diagnosis in systemic inflammatory patients [35].

4.2. suPAR. The soluble form of urokinase-type plasminogen activator receptor (suPAR) is a new biological marker of immunologic activation [36]. Urokinase-type plasminogen activator receptor (uPAR) is expressed on various cell types and participates in numerous immunologic functions including migration, adhesion, angiogenesis, fibrinolysis, and cell proliferation. uPAR/uPA system participated in migration of inflammatory cells from the bloodstream into tissues against infection. During inflammatory stimulation, uPAR is cleaved from the cell surface by proteases to create the soluble form of the receptor, suPAR, which can be detected in blood, urine, and cerebrospinal fluid [37]. Measurements can be obtained from commercial ELISA kits; suPAR measurements also are included in multiplex assays together with cytokines.

High serum suPAR concentrations have also been found to predict mortality in patients with active tuberculosis and other diseases associated with an inflammatory response [38].

Some studies have showed that suPAR levels were elevated in acutely ill patients but that their diagnostic value

Table 2: Evaluation of new biomarkers of sepsis.

New Biomarkers	Level	Sensit.	Specif.	AUC	NPV	PPV	Prevalence (%)	Type	Study
sTREM-1 (pg/mL)	40–755*	79	80	0.87	70	86	1113/1795 (62)	Diagnostic	[34]
Pro-ADM (nmol/L)	4.86	53	84	0.72	77	64	47/137 (34.7)	Prognostic	[44]
suPAR (ng/mL)	10	80	77	0.79	95	42	27/125 (21.6)	Diagnostic	[39]
	8.9	66	64	0.73	76	50	94/258 (36.43)	Diagnostic	[40]
Presepsin (pg/mL)	2866	79	62	0.70	87	45	55/189 (29)	Diagnostic	[47]
	1606	72	70	0.74	71	71	71/100 (71)	Prognostic	[48]
	317	71	86	0.82	52	93	372/859 (43.3)	Diagnostic	[49]
	556	62	67	nr	78	48	283/859 (32.94)	Prognostic	[49]

Sensit.: sensitivity, specif: specificity, AUC: area under curve, NPV: negative predictive value, PPV: positive predictive value, and nr: not reported.
*Cutoff point based in a meta-analysis of 11 studies.

was not superior to other biomarkers such as CRP, PCT, or sTREM-1 [39]. Recently, two studies evaluating diagnostic accuracy of suPAR have shown specificity from 64–77% [40, 41].

4.3. Pro-ADM. Adrenomedullin (ADM) is a 52-amino-acid peptide with immune modulating, metabolic, and vasodilator activity. Its widespread production in the tissues helps to maintain a blood supply in every organ. Moreover, ADM has a bactericidal activity and could be helpful in the evaluation of sepsis diagnosis and prognosis and in monitoring such conditions [42]. Prohormone fragments (pro-ADM) are more stable than the complete peptide and their levels can be measured in biological fluids by automated methods using the TRACE (Time-Resolved Amplified Cryptate Emission) method after immunocapture. The midregional fragment of proadrenomedullin (MR-pro-ADM), included between amino acids 45–92, is the most stable part of the ADM, and it has been detected in plasma of patients with septic shock as a consequence of the ADM active peptide degradation [43].

Pro-ADM is a biomarker of prognostic value and could be used to identify more severe patients with pneumonia and/or needing ICU care [44].

In a recent single prospective observational study conducted in a Spanish adult intensive care unit (137 patients), pro-ADM showed a significant dose-response trends to predict hospital mortality (OR = 3.00, 95% CI 1.06–8.46) compared to PCT and CRP. However, the prognostic accuracy was better for severity scores than for any biomarker [45].

In an Italian study comparing PCT and MR-pro-ADM in 200 septic patients, 90 patients with SIRS, and 30 healthy individuals, the pro-ADM distinguished septic patients. Moreover, the combined use of PCT and MR-pro-ADM gave a posttest probability of 0.998 in the cohort of all septic patients. The combination of biomarkers may substantially improve the early diagnosis of sepsis [46].

4.4. Presepsin. Cluster of differentiation 14 (CD14) is a glycoprotein expressed on the membrane surface of monocytes and macrophages and serves as a receptor for lipopolysaccharides (LPSs) and LPS-binding proteins (LPBs). By activating a proinflammatory signaling cascade on contact with infectious agents, CD14 has a role as a recognition molecule in the innate immune response against microorganisms. During inflammation, plasma protease activity generates soluble CD14 (sCD14) fragments. One of them, called sCD14 subtype (sCD14-ST), or presepsin, is normally present in very low concentrations in the serum of healthy individuals and has been shown to be increased in response to bacterial infections [47]. Plasma levels of presepsin can be measured using an automated chemoluminescent assay (PATHFAST).

In a multicenter prospective study (106 patients with suspected sepsis or septic shock were included and 83 SIRS patients without infection), elevated concentrations of presepsin were observed in septic patients compared to control patients [48]. The best diagnostic cutoff for presepsin was 600 pg/mL with sensitivity of 78.95% (95% CI, 69.4 to 86.6) and specificity of 61.90% (95% CI, 50.7 to 72.3). There was no difference between levels of presepsin and sepsis severity. Moreover, the area under the curve (AUC) calculated for PCT was wider, demonstrating a better diagnostic accuracy than presepsin. Although presepsin showed a significant prognostic value and initial values were significantly correlated with in-hospital mortality of patients affected by sepsis, severe sepsis, or septic shock, two recent studies have shown that presepsin is an useful biomarker for early diagnosis of sepsis and evaluation of prognosis in septic patients (sensitivity: 71-72%, specificity: 70–86%, and NPV: 52–71%) [49, 50].

5. Conclusions

(1) Bloodstream infection is a serious life-threatening condition with high mortality. In some cases, the diagnosis is challenging. An early diagnosis of sepsis helps to enable rapid treatment, improve outcomes, and reduce unnecessary antibiotic therapy.

(2) Choosing the correct empiric therapy is sometimes a difficult process. The emergence of resistant pathogens is consequence of irrational use of antibiotics.

(3) PCT and PCR are widely used in clinical practice and are more useful to rule out infection. PCT is the most studied biomarker that guides early stopping of antibiotic therapy in adults.

(4) New biomarkers are being evaluated in different clinical scenarios, although none of them have shown

sufficient sensitivity or specificity to rule out infection.

(5) Presepsin appears to be the most promising new biomarker for early diagnosis of sepsis and better prognostic performance than procalcitonin.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Research Article

Potential Synergy Activity of the Novel Ceragenin, CSA-13, against Carbapenem-Resistant *Acinetobacter baumannii* Strains Isolated from Bacteremia Patients

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Carbapenem-resistant *Acinetobacter baumannii* is an important cause of nosocomial infections, particularly in patients in the intensive care units. As chronic infections are difficult to treat, attempts have been made to discover new antimicrobials. Ceragenins, designed to mimic the activities of antimicrobial peptides, are a new class of antimicrobial agents. In this study, the in vitro activities of CSA-13 either alone or in combination with colistin (sulphate), tobramycin, and ciprofloxacin were investigated using 60 carbapenem-resistant *A. baumannii* strains isolated from bacteremia patients blood specimens. MICs and MBCs were determined by microbroth dilution technique. Combinations were assessed by using checkerboard technique. The MIC_{50} values (mg/L) of CSA-13, colistin, tobramycin, and ciprofloxacin were 2, 1, 1.25, and 80, respectively. The MIC_{90} (mg/L) of CSA-13 and colistin were 8 and 4. The MBCs were equal to or twice greater than those of the MICs. Synergistic interactions were mostly seen with CSA-13-colistin (55%), whereas the least synergistic interactions were observed in the CSA-13-tobramycin (35%) combination. No antagonism was observed. CSA-13 appears to be a good candidate for further investigations in the treatment of *A. baumannii* infections. However, future studies should be performed to correlate the safety, efficacy, and pharmacokinetic parameters of this molecule.

1. Introduction

Acinetobacter baumannii is a Gram-negative coccobacillus that recently has become one of the most common and highly antibiotic resistant pathogens throughout the world, and it is associated with high rates of morbidity and mortality [1, 2]. The most common clinical manifestations of *A. baumannii* infections in the intensive care units (ICUs) are ventilator associated pneumonia (VAP) and bacteremia, which are associated with morbidity and mortality rates as high as 52% [3, 4]. Invariably, one of the most alarming characteristics of this microorganism is its ability to manifest resistance to all available antibiotics including carbapenems, which is even higher than in other Gram-negative bacilli included in the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) [5]. Multidrug-resistant (MDR) *A. baumannii* is a growing threat that leaves few therapeutic options and recently there has been a dramatic increase in carbapenem resistance in *A. baumannii*. The mechanisms of resistance to antimicrobials are principally acquired through its ability to exchange genetic material. This attribute makes the treatment of *A. baumannii* infections particularly difficult, especially in certain types of infections [6]. The lack of new antibiotics to treat MDR *A. baumannii* infections has led the Infectious Disease Society of America (IDSA) to describe *A. baumannii* as "an emblematic case of the mismatch between unmet medical needs and the current antimicrobial research and development pipeline" [7].

As chronic infections are difficult to treat, attempts have been made to discover new antimicrobial agents targeting novel sites that may circumvent resistance. One frequently studied target is the bacterial membrane. Most antimicrobial peptides display broad-spectrum antibacterial activities and

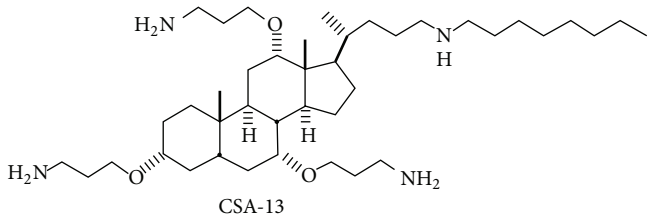
target the bacterial membrane. However, many antimicrobial peptides are difficult to synthesize and purify due to their complexity and size [8]. In addition, antimicrobial peptides can be substrates for proteases, which limit their in vivo half-lives [9]. Consequently, development of nonpeptide mimics of antimicrobial peptides may provide a means of using the antimicrobial strategies evolved over eons without the disadvantages of peptide therapeutics.

Recently, a series of cationic derivatives of cholic acid have been synthesized and have been found to have properties that may make them useful antimicrobial agents. The ceragenins, designed to mimic the activities of antimicrobial peptides, are a new class of antimicrobial agent. Ceragenins are not peptide based, are not salt sensitive, and are relatively simple to prepare and purify on a large scale [10]. Among them, CSA-13, which stands for cationic steroidal antimicrobial, is a lead ceragenin and is highly active against Gram-positive and Gram-negative bacteria. MIC determinations against common Gram-positive and Gram-negative bacteria have demonstrated that CSA-13 displays a broad spectrum of activity. CSA-13 displays antimicrobial activity against vancomycin-resistant *Staphylococcus aureus* [11], *Pseudomonas aeruginosa* [12, 13], *Helicobacter pylori* [14], and periodontopathic bacteria such as *Streptococcus mutans* and *Porphyromonas species* [15]. CSA-13 is also active against vaccinia virus [16] and *Trypanosoma cruzi* [17]. In animal studies, CSA-13 shows low toxicity, supporting this compound's possible application in human treatment [18].

In the setting of increasing resistance and diminishing therapeutic options, the "old" antibiotic colistin (polymyxin E) is now being used more extensively, especially in *P. aeruginosa* and carbapenem-resistant *A. baumannii* infections [19]. There are no current published studies evaluating the interactions between CSA-13 and colistin against carbapenem-resistant *A. baumannii* strains isolated from blood specimens. Therefore, the purpose of this study was to evaluate the in vitro activities of CSA-13 alone and in combination with colistin, tobramycin, and ciprofloxacin against 60 carbapenem-resistant *A. baumannii* strains isolated from bacteremia patients' blood specimens.

2. Materials and Methods

2.1. Bacterial Isolates. A total of sixty nonrepeat, bloodstream strains of carbapenem-resistant *A. baumannii* recovered from bacteremia patients in the year 2010-2011 admitted to the various hospitals in Turkey were included in the study. Thirty of these strains are obtained from the Department of Infectious Diseases and Clinical Microbiology, Medipol University, Istanbul, twenty of them are obtained from the Department of Infectious Diseases and Clinical Microbiology, Faculty of Medicine, Istanbul University, Istanbul, and the rest of them are obtained from Canakkale Onsekiz Mart University, Faculty of Medicine, Canakkale, Turkey. All strains were identified by the API 20 NE System (bioMerieux Vitek, Marcy l'Etoile, France). Isolates were defined as carbapenem-resistant strains using the disc diffusion and microdilution method. For the checkerboard experiments totally 20 strains from the three different institutions were used, since we carried out the combination experiments with susceptible strains. *Escherichia coli* ATCC 25922 (Rockville, Md., USA) was used as a quality control strain.



CSA-13

Figure 1: The chemical structure of ceragenin CSA-13 (molecular weight 822.94).

2.2. Antimicrobial Agents. CSA-13 was synthesized from a cholic acid scaffold technique as previously described (Figure 1) [20]. Colistin was obtained from Sigma Aldrich and tobramycin, ciprofloxacin, and meropenem were kindly provided from Bilim and Bayer Pharmaceuticals and Astra Zeneca, respectively. Stock solutions from dry powders were prepared in water and stored frozen at −80°C. Frozen solutions of antibiotics were used within 6 months.

2.3. Media. Mueller-Hinton broth (MHB, Difco Laboratories, Detroit, MI) supplemented with divalent cations to a final concentration of 25 mg of Mg^{2+} and 50 mg of Ca^{2+} per liter (CSMHB) was used for all the experiments. Pour plates of Tryptic Soy agar (TSA, Difco Laboratories, Detroit, MI) were used for colony counts.

2.4. Determinations of MICs and MBCs. MICs were determined by the microbroth dilution technique as described by CLSI [21, 22]. Serial twofold dilutions ranging from 256 to 0.25 mg/L were prepared in CSMHB. The inoculum was prepared with a 4–6 h broth culture that gives a final concentration of 5×10^5 cfu/mL in the test tray. Experiments were performed in duplicate. MBCs were determined at the conclusion of the incubation period by removing two 0.01 mL samples from each well demonstrating no visible growth and plated onto TSA. The MBC was defined as the lowest concentration of antibiotic giving at least a 99.9% killing of the initial inoculums [23].

2.5. Determination of Fractional Inhibitory Concentration Index (FICI). The effects of antibiotics in combination were assessed by using the microbroth checkerboard technique [24]. Each microtiter well containing the mixture of antibiotics was inoculated with a 4–6 h broth culture diluted to give a final concentration of approximately 5×10^5 cfu/mL. After incubation at 37°C for 18–20 h the fractional inhibitory concentration index (FICI) was determined as the combined concentration divided by the single concentration. The combination value was derived from the highest dilution of antibiotic combination permitting no visible growth. With this method, synergy was defined as a FICI of ≤0.5, no

Table 1: Comparative in vitro activity of antimicrobial agents against 60 isolates of *A. baumannii*.

Antibiotics	mg/L						Percent inhibited at CLSI breakpoints[a]		
	MIC range	MIC_{50}	MIC_{90}	MBC range	MBC_{50}	MBC_{90}	Susceptible	M.S.[b]	Resistant
CSA-13	1–16	2	8	1–32	2	16	—	—	—
Colistin	0.06–32	1	4	0.06–32	2	8	86	0	14
Tobramycin	0.3–160	1.25	80	0.3–160	2.5	160	45	0	55
Ciprofloxacin	0.3–80	80	160	0.6–160	80	160	5	0	95
meropenem	16–128	32	64	16–256	64	128	0	0	100

[a]CLSI breakpoints for susceptible and resistant to colistin ≤2 mg/L and ≥4 mg/L; tobramycin ≤4 mg/L and ≥16 mg/L; ciprofloxacin ≤1 mg/L and ≥4 mg/L and meropenem ≤4 mg/L and ≥16 mg/L, respectively.
[b]M.S.: moderately susceptible.

Table 2: In vitro activity of CSA-13 and colistin combined with studied antibiotics against *A. baumannii* strains.

Antibiotic combinations	*n*	Number (%) of synergistic effects
CSA-13 + colistin	20	11 (55)
CSA-13 + tobramycin	20	7 (35)
CSA-13 + ciprofloxacin	20	8 (40)
colistin + tobramycin	20	9 (45)
colistin + ciprofloxacin	20	9 (45)

interaction as a FICI of >0.5–4, and antagonism as a FICI of 4.0 [25].

3. Results

3.1. Susceptibility. The in vitro activities of the studied antibiotics against 60 *A. baumannii* strains are summarized in Table 1. Susceptibility testing demonstrated that the MIC ranges for CSA-13, colistin, tobramycin, and ciprofloxacin were 1–16, 0.06–32, 0.3–160, and 0.3–80 mg/L and MBC ranges for those antibiotics were 1–32, 0.06–32, 0.3–160, and 0.6–160 mg/L, respectively. As seen from the results, CSA-13 showed a similar pattern of MIC and MBC ranges as colistin. In addition, the highest MIC and MBC values of CSA-13 were just onefold higher of the MIC_{90} and MBC_{90} values. However, 14%, 55%, and 95% of the strains were found resistant to colistin, tobramycin, and ciprofloxacin, respectively. All the strains were resistant to meropenem. CSA-13 MICs (and also MBCs) of the colistin-resistant strains are at the same value or twofold greater than those of the colistin-resistant strains. There was no major difference between bactericidal and inhibitory endpoints. The MBCs were generally equal to or twofold greater than those of the MICs.

3.2. Checkerboard. The results of combination studies are shown in Table 2. With a FIC index of ≤0.5 as borderline, synergistic interactions were mostly seen with CSA-13-colistin combination (synergism was observed with 55% of the strains tested), whereas the least synergistic interactions were observed with the CSA-13-tobramycin combination (synergism was observed with 35% of the strains tested). No antagonism was observed with any combination.

4. Discussion

Ceragenins are a group of cholic acid derivatives that have potent activities against various microorganisms [10]. Here, we report an MIC_{50} of 2 mg/L for CSA-13 against 60 carbapenem-resistant *A. baumannii* strains. As seen from the results, MIC_{90} value of CSA-13 was equal to two dilutions higher of the MIC_{50} value, which is parallel to colistin results (Table 1). These results indicate that CSA-13 shows an activity with similar MIC values independent of whether or not the bacteria are resistant to other antibiotics. Probably, this situation could be attributed to its ability to permeabilize both outer and cytoplasmic membrane of the bacteria and its resistance to protease degradation [26]. These results support the idea that development of resistance to CSA-13 might be rare if it is used in the treatment. Our study also shows that CSA-13 has an MIC_{50}/MBC_{50} ratio of 1, suggesting that the bactericidal activity is close to the inhibitory concentration. Indeed, varying CSA-13 concentrations at, below, and above the MIC demonstrated rapid bactericidal antimicrobial activity, even when the strains were resistant to colistin, ciprofloxacin, and/or tobramycin, similar to our previous work [13].

Carbapenem resistance rates are increasing to such an extent to threaten the world and this situation is becoming a routine phenotype for the *A. baumannii*. Therefore, in order to take the microorganism under control, selection of the antimicrobial agents is extremely important [27]. Increase of carbapenem resistance raises the fact that the reuse of old antibiotics like polymyxin E. (colistin) has a lower rate of mortality than carbapenems in treatment of multidrug resistance infections [19]. Colistin is frequently used to treat infections caused by carbapenem-resistant *A. baumannii*, due to its efficacy [28]. However, recently colistin resistance is reported worldwide, especially in Europe [29, 30]. In our country, Ergin et al. reported colistin resistance as 2% in *A. baumannii* [31]. According to our study, 86% of the strains were found to be colistin susceptible. Moreover, we demonstrated that of all the studied antibiotics, colistin was active at the lowest MIC (MIC_{50} = 1 μg/mL) value against the strains. This may arise from the fact that colistin has been recently used for clinical use in Turkey.

Results of our study showed that the highest resistance rate is obtained with ciprofloxacin (95%). This was in accordance with one multicentric study [32]. High percentages

of strains belonging to *A. baumannii* were resistant to ciprofloxacin, ofloxacin, and cefotaxime (79, 76, and 54%, resp.) by agar dilution method.

Management of the infections caused by carbapenem-resistant *A. baumannii* is difficult and combination therapy for the treatment of carbapenem-resistant *A. baumannii* has increasingly been used [28]. Therefore, in our study, in vitro interactions of CSA-13 in combination with colistin, tobramycin, and ciprofloxacin against carbapenem-resistant *A. baumannii* strains were assessed by using the microbroth checkerboard technique since it provides fast results and interpretation of these results is simple [24]. The results of this in vitro trial provide evidence that, with a FICI of ≤0.5 as borderline, synergistic interactions were detected in all combinations (Table 2). Synergistic interactions were mostly seen with CSA-13-colistin combination (55% of tested strains), whereas the least synergistic interactions were observed with the CSA-13-tobramycincombination (35% of tested strains).

Consequently, ceragenins are novel molecules resistant to proteolysis and promise opportunities in treatment of bacterial, fungal, and even viral infections. According to the results of this in vitro study, CSA-13 may have important therapeutic implications for infections caused by carbapenem-resistant *A. baumannii* strains. So, these molecules should be evaluated carefully and must be reserved for the most important necessity. Possible success for the combination therapy of ceragenins and colistin or other antibiotics depends on the pharmacokinetics and pharmacodynamics of these molecules in vivo. Therefore, future studies should be performed to correlate the safety, efficacy, and pharmacokinetic parameters of these combinations.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Aetiology of Bacteraemia as a Risk Factor for Septic Shock at the Onset of Febrile Neutropaenia in Adult Cancer Patients

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Septic shock (SS) at the onset of febrile neutropaenia (FN) is an emergency situation that is associated with high morbidity and mortality. The impact of the specific aetiology of bloodstream infections (BSIs) in the development of SS at the time of FN is not well established. The aim of this study was to evaluate the association between the aetiology of BSIs and SS at the time of FN in hospitalised adult cancer patients. This prospective cohort study was performed at a single tertiary hospital from October 2009 to August 2011. All adult cancer patients admitted consecutively to the haematology ward with FN were evaluated. A stepwise logistic regression was conducted to verify the association between the microbiological characteristics of BSIs and SS at the onset of FN. In total, 307 cases of FN in adult cancer patients were evaluated. There were 115 cases with documented BSI. A multivariate analysis showed that polymicrobial bacteraemia ($P = 0.01$) was associated with SS. The specific blood isolates independently associated with SS were viridans streptococci ($P = 0.02$) and *Escherichia coli* ($P = 0.01$). Neutropaenic cancer patients with polymicrobial bacteraemia or BSI by viridans streptococci or *Escherichia coli* are at increased risk for SS at the time of FN.

1. Introduction

Despite improvements in treating febrile neutropaenia (FN) and sepsis over the past decade, septic shock (SS) continues to be associated with substantial morbidity and mortality among cancer patients undergoing intensive cytotoxic chemotherapy [1]. The unpredictable clinical course of infections in neutropaenic patients because of the lack of an adequate inflammatory response makes managing FN a significant challenge because clinically stable patients may suddenly progress to severe sepsis or SS [2].

SS is a result of the host response to the pathogen and is dependent on the virulence of the microorganism and the infection site [3]. The known risk factors for SS in immunocompetent patients include advanced age, low functional status, and the presence of cancer, clinical comorbidities, nosocomial infections, and infection that does not originate in the urinary tract [4–6]. Infection with certain bacteria, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, is also associated with an increased risk for SS, as the expression of certain proteins or molecules (virulence factors) contributes to pathogen replication and dissemination by subverting or eluding the host's defences [7]. Unfortunately, data regarding the influence of microbiological factors on the development of SS in cancer patients with FN is scarce. Therefore, we conducted a study with the aim of evaluating the association between microbiological aspects of bloodstream infections (BSIs) and SS development at the onset of FN in hospitalised adult cancer patients.

2. Methods

2.1. Study Design and Participants. A prospective cohort study was conducted at a single referral centre for adult bone marrow transplantation in Southern Brazil from October 2009 to August 2011. This study followed all consecutive haemodynamically stabile cancer patients older than 18 years of age who were admitted to the haematology ward of the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) with neutropaenia (i.e., an absolute neutrophil count (ANC) < 500 cells/mm^3 or <1000 cells/mm^3 with an expectation of a decrease to <500 cells/mm^3 during the ensuing 48 h).

The subjects who developed fever (i.e., a single axillary temperature measurement ≥38.5°C or sustained temperature ≥38.0°C over a 1 h period) during the course of neutropaenia were entered into the study. Outpatients, patients who had neutropaenia caused by a specific aetiology other than an adverse reaction to chemotherapy, and patients who had episodes of FN without documented bacteraemia were excluded. Subjects were allowed to reenter the study after an initial episode of FN if they remained free of signs or symptoms of infection for at least 7 days after completing the treatment for the first episode and if all causative organisms, if any, were eradicated.

2.2. Definitions. Microbiological studies, which included 2 separate blood samples that were obtained from 2 different anatomical sites for culture, were performed at the onset of fever, according to standard practice. In the absence of an indwelling central venous catheter, 2 blood samples were obtained from 2 distinct peripheral veins. When an indwelling central venous catheter was present, 1 blood sample was obtained through this catheter, and a second sample was obtained from a peripheral vein. The susceptibilities of the isolated pathogens to antibiotics were evaluated according to the recommendations of the Clinical and Laboratory Standards Institute [8]. Bacteraemia caused by coagulase-negative *Staphylococcus* spp. was diagnosed after 2 positive results from 2 independent cultures. Bacteraemia indicated by 1 positive culture was considered to be diagnostic for the other microorganisms. Polymicrobial BSI was characterised as a bacteraemic episode due to at least two different pathogens isolated from the same blood sample. Multidrug-resistant (MDR) bacteraemia was defined as a BSI with methicillin-resistant staphylococci or vancomycin-resistant enterococci for Gram-positive bacteria or as resistance to ≥3 classes of antimicrobial agents for Gram-negative bacteria. Clinical comorbidity was determined by the presence of heart failure, diabetes mellitus, chronic pulmonary disease, chronic liver disease, or chronic renal failure. Profound neutropaenia was characterised by an ANC < 100 cells/mm^3. The patients were divided into 2 groups based on their chemotherapy regimens: a high-dose chemotherapy group that included patients who underwent haematopoietic stem cell transplantation or induction chemotherapy and a standard-dose chemotherapy group that included patients who underwent consolidation or maintenance chemotherapy. Nosocomial-acquired FN was defined as the onset of FN after 48 hours of hospitalisation. The oral mucositis grade was classified according to the World Health Organisation's oral toxicity scale [9].

2.3. Outcomes and Followup. The primary outcome measure of the present study was SS at the onset of fever in neutropaenic patients. SS was defined as persistent haemodynamic instability (systolic arterial pressure <90 mmHg or a reduction in systolic blood pressure >40 mmHg from baseline) despite adequate fluid resuscitation (30 mL per Kg of crystalloid) with at least 2 systemic inflammatory response syndrome criteria [10]. The secondary outcome was mortality by day 28. Researchers who were not associated with the assistant physician's team conducted the patient followups through interviews and medical record reviews using a standardised data collection instrument. The followup was maintained for 28 days after the onset of fever in the neutropaenic patients. For the subjects who were discharged before 28 days, follow-up telephone calls were made on the 28th day after the onset of FN to determine whether they remained alive; if a patient was deceased at the time of the phone call, the survival time was calculated based on the date of death reported by the family.

2.4. Statistical Analysis. Stepwise logistic regression analysis was performed to determine whether the microbiological characteristics of BSIs were risk factors for SS at the time of FN. All clinical and microbiological variables that had a P value <0.10 in the univariate analysis were included. In the multivariate model, independent variables were eliminated from the highest to the lowest P value but remained in the model if the P value was <0.05. Odds ratios (OR) were estimated with 95% confidence intervals (95% CI). Kaplan-Meier curves were utilised to evaluate the time-dependent occurrence of death; the log-rank test was applied for between-group comparisons. The statistical analysis was performed using STATA version 12 (Stata Corp LP, USA).

2.5. Ethics Statement. Written informed consent was obtained from all study participants. The institutional review board of the Hospital de Clínicas de Porto Alegre approved the study.

3. Results

In total, 307 episodes of FN (in 169 patients) were evaluated; a total of 115 BSIs were documented (37.4% of all episodes). Antibiotic prophylaxis was not administered to any patient. The incidence of SS was 14.7% (17 episodes).

The characteristics of the study population and the specific pathogens responsible for all BSIs in the present cohort are shown in Table 1. Subjects with haematological malignancies comprised 83.5% of the study population; haematopoietic stem cell transplantation was performed in 21.7% of the cases. Forty-eight percent of the study sample had some degree of chemotherapy-induced mucositis. The proportion of nosocomial episodes of FN was 88.7%. In descending order, the predominant blood isolates were *Escherichia coli*, coagulase-negative staphylococci, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, viridans streptococci, and *Enterococcus* spp. Among all BSIs evaluated, 38 cases were due to MDR bacteria (Table 2): 4 cases in the SS group (23.5%) and 34 cases in the non-SS shock group (34.6%). Methicillin resistance and the production of extended-spectrum beta-lactamase were the most frequent types of antimicrobial resistance, occurring in 96.2% of BSIs involving Gram-positive MDR bacteria and 83.3% of BSIs involving Gram-negative MDR bacteria, respectively.

A univariate analysis revealed that polymicrobial BSI ($P = 0.01$) and bacteraemia by *Escherichia coli* ($P = 0.04$) were associated with the main outcome (Table 3). Multidrug-resistant (MDR) bacteraemia was not associated with SS at

Table 1: Study population characteristics and microorganisms isolated in 115 cases of febrile neutropenia (FN) in hospitalised cancer patients with documented bloodstream infection.

Age, mean years ± SD	42.9 ± 14.1
Female sex	52 (45.2)
Type of cancer	
Acute myeloid leukaemia	59 (51.3)
Acute lymphoblastic leukaemia	19 (16.5)
Chronic myeloid leukaemia	7 (6.1)
Multiple myeloma	11 (9.6)
Lymphoma	15 (13.0)
Other solid tumours	4 (3.5)
Relapsing underlying disease	59 (51.3)
Clinical comorbidity	36 (31.3)
Phase of chemotherapy	
Induction	27 (23.5)
Consolidation	37 (32.2)
Maintenance	26 (22.6)
HSCT	25 (21.7)
Oral mucositis	
Without oral mucositis	59 (51.3)
Grade I	33 (28.7)
Grade II	10 (8.7)
Grade III	6 (5.2)
Grade IV	7 (6.1)
ANC at the time of diagnosis of FN, mean cells/mm^3 ± SD	206.1 ± 218.5
Profound neutropenia at the time of diagnosis of FN*	52 (45.2)
Nosocomial-acquired episodes of FN	102 (88.7)
Bloodstream isolates†	
Escherichia coli	48 (41.7)
Coagulase-negative staphylococci	36 (31.3)
Klebsiella pneumonia	13 (11.3)
Pseudomonas aeruginosa	11 (9.5)
Viridans streptococci	8 (6.9)
Enterococcus spp.	4 (3.4)
Serratia spp.	2 (1.7)
Enterobacter spp.	2 (1.7)
Candida spp.	2 (1.7)
Salmonella spp.	1 (0.8)
Staphylococcus aureus	1 (0.8)
Kocuria varians	1 (0.8)

Data presented as *n* (%) unless otherwise indicated. SD: standard deviation; ANC: absolute neutrophil count; HSCT: hematopoietic stem cell transplantation; *ANC < 100 cells/mm^3; †There were 12 cases of polymicrobial bloodstream infections.

the onset of FN with either the Gram-positive MDR or Gram-negative MDR bacteria.

After multiple logistic regression analyses were performed (Table 4, model 1), the only variable that constituted an independent risk factor for SS at the time of FN was polymicrobial BSI (OR, 5.41, 95% CI, 1.48–19.79). A second logistic regression model was used to assess the effect of specific pathogens on the development of SS without the inclusion of other microbiological variables (Table 4, model 2). This model was conducted to avoid the dilution of the effect of specific pathogens by other microbiological factors in the multivariate analysis. The specific blood isolates that were independently associated with the main outcome were viridans streptococci (OR, 7.58, 95% CI, 1.34–42.80) and *Escherichia coli* (OR, 4.30, 95% CI, 1.34–14.48). The percentage of the polymicrobial samples that included *E. coli* and viridans streptococci was 58.3% (7 cases) and 25% (3 cases), respectively.

As expected, the 28-day mortality rate of the patients who presented with SS at the time of FN was greater than that of the patients who did not present with SS (35.2% versus 14.2%, log-rank $P = 0.01$) (Figure 1).

Table 2: Multidrug-resistant bacteria isolated in 38 cases of bacteraemia in febrile neutropenic patients.

Microorganism	Number isolated (%)
Gram-positive	
MR coagulase-negative staphylococci	25 (65.7)
MR *Staphylococcus aureus*	1 (2.6)
VR *Enterococcus faecalis*	1 (2.6)
Gram-negative	
Escherichia coli ESBL	7 (18.4)
Klebsiella pneumoniae ESBL	3 (7.8)
Enterobacter spp.	1 (2.6)
Serratia spp.	1 (2.6)

MR: methicillin resistant; VR: vancomycin resistant; ESBL: extended-spectrum beta-lactamase. There was 1 case of polymicrobial multidrug-resistant bacteraemia.

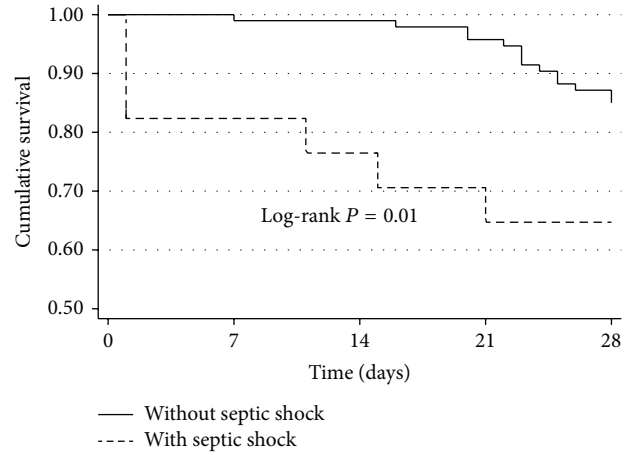

Figure 1: Survival curves according to the presence of septic shock at the time of febrile neutropenia in hospitalised adult cancer patients.

4. Discussion

The present prospective cohort study demonstrated that cancer patients with polymicrobial bacteraemia were more

Table 3: Univariate analysis of the risk factors for septic shock (SS) at the time of febrile neutropenia (FN) in hospitalised cancer patients.

Variable	SS group (n = 17)	Non-SS group (n = 98)	OR (95% CI)	P value
Age, years, mean ± SD	43.4 ± 16.0	42.8 ± 13.8	1.00 (0.96–1.04)	0.87
Female sex	10 (58.8)	42 (42.8)	1.90 (0.66–5.41)	0.22
Haematological neoplasm	13 (76.4)	83 (84.6)	0.58 (0.16–2.04)	0.40
Relapsing underlying disease	10 (58.8)	49 (50.0)	1.42 (0.50–4.05)	0.50
Clinical comorbidity	2 (11.7)	34 (34.6)	0.25 (0.05–1.16)	0.07
High-dose chemotherapy regimens*	5 (29.4)	47 (47.9)	0.45 (0.14–1.38)	0.16
Oral mucositis				
Grade I	4 (23.5)	29 (29.5)	0.76 (0.21–2.71)	0.68
Grade II	2 (11.7)	8 (8.1)	1.38 (0.25–7.63)	0.70
Grade III	1 (5.8)	5 (5.1)	1.11 (0.11–10.66)	0.92
Grade IV	1 (5.8)	6 (6.1)	0.92 (0.09–8.63)	0.94
ANC at the time of diagnosis of FN, mean ± SD	161.7 ± 219.0	213.8 ± 218.7	0.99 (0.99–1.00)	0.36
Profound neutropenia at the time of diagnosis of FN†	9 (52.9)	43 (43.8)	1.43 (0.51–4.04)	0.49
BSI involving Gram-positive bacteria	6 (39.2)	40 (40.8)	0.79 (0.27–2.31)	0.66
BSI involving Gram-negative bacteria	14 (82.3)	60 (61.2)	2.96 (0.79–10.97)	0.10
Polymicrobial BSI	5 (29.4)	7 (7.1)	5.41 (1.48–19.79)	0.01
MDR BSI	4 (23.5)	34 (34.6)	0.57 (0.17–1.91)	0.37
BSI involving Gram-positive MDR bacteria	3 (17.6)	24 (24.4)	0.66 (0.17–2.49)	0.54
BSI involving Gram-negative MDR bacteria	1 (5.8)	11 (11.2)	0.49 (0.05–4.09)	0.51
BSI by *Escherichia coli*	11 (64.7)	37 (37.7)	3.02 (1.03–8.85)	0.04
BSI by coagulase-negative staphylococci	4 (23.5)	32 (32.6)	0.63 (0.19–2.10)	0.45
BSI by *Klebsiella pneumoniae*	3 (17.6)	10 (10.2)	1.88 (0.46–7.70)	0.37
BSI by *Pseudomonas aeruginosa*	2 (11.7)	9 (9.1)	1.31 (0.25–6.70)	0.73
BSI by viridans streptococci	3 (17.6)	5 (5.1)	3.98 (0.85–18.54)	0.07

Data presented as n (%) unless otherwise indicated. ANC: Absolute neutrophil count; BSI: bloodstream infection; HSCT: haematopoietic stem cell transplantation; MDR: multidrug-resistant; OR: odds ratio; 95% CI: 95% confidence interval; SD: standard deviation. *Induction chemotherapy or HSCT; †ANC < 100 cells/mm^3.

Table 4: Multiple logistic regression analysis of the risk factors for septic shock (SS) at the time of febrile neutropenia (FN) in hospitalised adult cancer patients.

Risk factor	Adjusted OR	95% CI	P value
	Model 1		
Polymicrobial BSI	5.41	1.48–19.79	0.01
	Model 2		
BSI by *Escherichia coli*	4.30	1.27–14.48	0.01
BSI by viridans streptococci	7.58	1.34–42.80	0.02

OR: odds ratio; 95% CI: 95% confidence interval; BSI: bloodstream infection.

likely to develop SS at the onset of FN. In particular, BSIs involving *E. coli* and viridans streptococci were independently associated with SS at the time of FN. The 28-day survival rate of the patients with SS at the time of FN was significantly lower than that of the patients who did not present with SS.

Previous observational studies involving distinct populations have confirmed the influence of microbiological aspects of BSIs on the hazards of SS. Consistent with the results of our study, Leibovici et al. conducted a retrospective study involving more than 4000 episodes of bacteraemia in a general population and found that the polymicrobial aetiology was predictive of SS [11]. Moreover, the association of specific BSI by *E. coli* and viridans streptococci with SS in FN patients is feasible because invasive infections by *E. coli* and viridans streptococci are often associated with significant morbidity and mortality [12–15]. For example, the study of Marron et al. [15] reported an association between viridans streptococcal bacteraemia and serious complications, such as SS and acute respiratory distress syndrome, in neutropaenic patients receiving high-dose chemotherapy with cyclophosphamide before allogeneic bone marrow transplantation.

Interestingly, none of the studied clinical characteristics was significantly associated with SS at the time of FN in the multiple logistic regression analysis. These findings differ from the immunocompetent patient studies in which the early development of SS was more frequent in the subjects with advanced age and multiple comorbidities [4–6]. One possible explanation is the relative homogeneity of our study population, which consisted of a large proportion of young patients with haematological malignancies and a relatively low prevalence of associated comorbidities. This fact highlights the need to identify the rapidly available clinical diagnostic features that can predict septic shock in this setting.

This study had some limitations. For example, we found a low incidence of bacteraemia by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which are often associated with a poorer prognosis in septic patients; therefore, our results should be interpreted with caution, as distinct virulent

bacteria may be found in other centres. Furthermore, this study was susceptible to biases that are inherent to observational studies; however, the following factors minimised the possibility of systematic errors: the proper measurement of variables and outcomes with previously defined objective criteria, the use of standardised data collection, the implementation of a followup by a research team that was not related to the care provided, and the use of multivariate analyses.

5. Conclusions

The aetiology of BSIs is an important risk factor for SS at the onset of FN in adult cancer patients. Polymicrobial BSI, particularly bacteraemia by *E. coli* and viridans streptococci, are the risk factors for SS at the onset of FN.

Identifying the microbiological factors associated with SS in FN is of paramount importance to clinicians, as this knowledge can determine the preventative measures to avoid BSI by specific highly virulent pathogens and the best choice of empiric antimicrobial therapy.

Future studies are required to assess other possible risk factors for the early onset of SS in the context of FN and to determine whether specific interventions based on the early identification of highly virulent bacteria could result in an effective method to prevent SS and its characteristically pronounced mortality rates.

Conflict of Interests

The authors declare no conflict of interests. This work was partially supported by CNPq (Brazilian National Council of Research).

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Review Article

Biomarkers for Sepsis: A Review with Special Attention to India

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Sepsis is a serious infection and still a common cause of morbidity and mortality in resource-limited settings such as India. Even when microbiologic diagnostics are available, bacteremia is only identified in a proportion of patients who present with sepsis and bloodstream infections. Biomarkers have been used in a variety of disease processes and can help aid in diagnosing bacterial infections. There have been numerous biomarkers investigated to aid with diagnosis and prognostication in sepsis with the majority suffering from lack of sensitivity or specificity. Procalcitonin has been heralded as the biomarker that holds the most promise for bloodstream infections. Data are emerging in India, and in this review, we focus on the current data of biomarkers in sepsis with particular attention to how biomarkers could be used to augment diagnosis and treatment in India.

1. Introduction

Sepsis and its complications are a common cause of infectious disease illness and mortality worldwide [1] and are a significant contributor to child death in India [2, 3]. Consensus definitions of sepsis were first published in 1992 [4] and later updated [5]. Better understanding of the pathophysiology of sepsis, new diagnostics, and improved therapeutics were reviewed in the surviving sepsis campaign guidelines [6] and subsequently revised [7]. International guidelines were published, and these have been supported and published in Indian medical journals [8].

Sepsis is defined as systemic inflammatory response syndrome (SIRS) caused by infection [7, 9]. However, infections can be difficult to confirm. Fever, tachycardia, hypotension, and other vital sign abnormalities found in SIRS are not specific for infection and overlap with noninfectious etiologies presenting with systemic inflammation. There is no gold standard for diagnosing infection, and though blood cultures processed with standard microbiologic techniques are a frequent diagnostic step, their likelihood of returning with the pathogen of interest depends on a variety of factors, including prior antibiotic therapy [10, 11]. Delays in empiric treatment for sepsis and bacteremia increase mortality [12] as well as length of stay [13] and cost [14], making timely recognition of infection and initiation of appropriate therapy an important goal. Standard blood culture techniques require time with results typically not available for at least 24–48 hours, highlighting the need for rapid diagnosis and risk stratification where biomarkers could be of use.

There have been many attempts to augment clinical decision making with diagnostic tests to increase sensitivity and specificity when diagnosing and treating sepsis and bacteremia. Initial studies employed fever and leukocytosis to define sepsis [4], though these tests were nonspecific. Subsequent studies focused on erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) to help in the diagnostic algorithm but suffered from the same lack of specificity. As our knowledge of sepsis evolved, it became evident that not only direct pathogen effects but also an exuberant inflammatory host response was responsible for the deleterious clinical and laboratory abnormalities. Sepsis is a systemic inflammatory syndrome affecting all organ systems, and biomarkers have focused on a number of pathogen and host responses, including cytokines, cell markers, receptor biomarkers, coagulation, vascular endothelial damage, vasodilation, organ

dysfunction, acute phase protein markers, and other systems. Sepsis provokes a systemic host response involving hundreds of mediators that could be potentially used as biomarkers for both diagnosis and prognosis [15]. A recent review detailed nearly 180 biomarkers that have been evaluated including IL-6, IL-8, lactate, soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), and procalcitonin (PCT) [16]. PCT has been the most studied and felt to hold the most promise.

India, with population of 1.2 billion [17], has one of the highest infectious disease burdens in the world [18]. While systemic data on presentations of acute febrile illness are lacking, 12% of adults (range 1–51%) of those presenting with acute febrile illness will have bacteremia [19]. While sepsis is not interchangeable with bloodstream infections, the majority of research has been done on sepsis as a syndrome and will be evaluated in this review. Availability of diagnostic assays is variable in India [20], making diagnosis of these common infections even more difficult. There is great interest in developing decision tools that utilize biomarkers to help aid the rapid diagnosis of bacterial infections. Additionally, due to rising antibiotic resistance on the Indian subcontinent, biomarkers that help with antibiotic stewardship are equally needed. There have been numerous studies evaluating PCT in different clinical scenarios, including sepsis, though the majority of these studies have been in the United States and Europe; there is great opportunity for well-designed studies evaluating biomarkers for sepsis in India.

2. WBC, ESR, Lactate, and CRP

Initial consensus statements focusing on sepsis definitions employed vital sign abnormalities and leukocytosis [4], but it is well recognized that overlapping with noninfectious etiologies exist [21]. Other routinely obtained, widely available tests such as lactate, serum glucose, and platelet counts that exhibit abnormalities in sepsis are nonspecific [9]. While leukocyte count was employed in initial definitions of sepsis, both leukocyte count and reliance on immature forms have low predictive value [22–24]. Lactate has been incorporated into definitions for sepsis, and normalization of serum lactate levels has been used as part of goal-directed care [25]. There is a broad consensus that an association between elevated lactate concentrations and poorer outcome is seen; however, a recent review [26] that included 28 studies found no ability to recommend a threshold value because of the extensive overlap of levels among patients with different outcomes. There is also the belief that elevated lactate levels occur later in disease and are less helpful as biomarkers from a diagnostic perspective because other signs, symptoms, or data will be available by that time. Serum lactate concentration at time of admission has been recommended by the surviving sepsis campaign guidelines as a marker of hypoperfusion [6] and a trial looked at using serum lactate to monitor resuscitation efforts [27].

ESR has long been used as an adjunctive test for inflammation; however, its utility as a biomarker for sepsis is limited [28]. There have been many studies comparing ESR with CRP; a recent study looked at the clinical utility of each test and variations in results stratified by age and concluded that each test provided similar information but that the time lapse for escalation and resolution was faster for CRP [29]. In this review, we will focus on CRP as it relates to sepsis. CRP is an acute-phase reactant produced only by hepatocytes in response to inflammation or tissue injury. In healthy young adult volunteer blood donors, the median concentration of CRP is below 0.8 mg/L and can increase 1,000-fold in response to an acute-phase stimulus [30, 31]. CRP hepatic synthesis starts rapidly after a stimulus with rise noted by about 6 hours with peak around 48 hours and a plasma half-life of approximately 19 hours. The half-life is constant under all conditions, so hepatic synthesis determines the serum concentration [32]. IL-6, as well as other cytokines, has been found to stimulate CRP production [31]. ESR and CRP have been known for a long time to be elevated in inflammatory conditions, including infection, and were used widely as an adjunctive test in sepsis and have often been used as a comparator for newer biomarkers [33]. Later studies have questioned their utility due to lack of specificity [34–36]. Studies have found that CRP changes were not influenced by neutropenia in septic patients [37], but CRP was not a good predictor of infection in neutropenic patients [38]. Elevated CRP levels in sepsis have been correlated with increased risk of death and organ failure [39], but in part due to the persistence of elevated levels, were unable to predict survival when evaluating CRP trends [40, 41]. CRP has been used successfully during initial sepsis diagnosis, but its specificity is further reduced later in the course due to persistently elevated levels [42]. CRP has been found to be significantly elevated in sepsis due to gram negative infections compared with gram positive infections suggesting a different immunomodulatory response [43].

A year-long study evaluating 57 episodes of febrile neutropenia among 26 young adults found that a rise in CRP on day 3 showed a significant difference between those with microbiologically defined infection when compared with fever without microbiologic diagnosis and was able to differentiate those that responded to addition of antifungal therapy and those that responded to second line antimicrobial therapy [44].

There have been several investigations of CRP in sepsis in India. Sugitharini et al. found that CRP levels were significantly elevated in neonates with sepsis compared with those without [45]. While Sugitharini's study did not report sensitivity of CRP in detecting sepsis, a study comparing CRP levels in 80 septic pediatric patients in India with 30 healthy pediatric controls found that CRP had a sensitivity of 67%, specificity 97%, PPV 98%, and NPV 53%; this study found a higher sensitivity and specificity for TNF-alpha levels (sensitivity 85%, specificity 100%, PPV 100%, and NPV 71%) [46]. While not specific to bacteremia, malaria is a common complicating factor in patients who appear septic in India which is not seen in locations that the majority of biomarker research has been conducted; researchers have found that CRP is elevated in cases of acute malaria [47] and degree of CRP elevated was correlated with death and length of hospitalization. CRP levels are known to be influenced by genetic variants in Europeans, and one study evaluated

genetic variants in Indian patients [48]. A study looking at nondiabetic Asian Indians living in the United States found significant elevation of plasma CRP levels [49]. More research will need to be done to see how this affects interpretation of CRP in Indian patients, though CRP will likely not be found to have the necessary discriminatory power for diagnosis and treatment of sepsis.

3. Procalcitonin

Procalcitonin is a 116 amino acid polypeptide precursor for the hormone calcitonin. It was first identified in 1975 and first linked to infectious disease in 1983 when increased serum levels of immunoreactive calcitonin were described in patients with staphylococcal toxic shock syndrome [50]. It was not until Assicot et al. reported high serum PCT levels in sepsis that the current research on PCT in bacterial disease accelerated [51]. Procalcitonin offers favorable kinetics for a biomarker: rising prior to two hours [52], reliably detectable between 2 and 4 hours, peaking at 6 hours, and maintaining a plateau through 8 and 24 hours [53]. At physiologic homeostasis, PCT is detectable in very low levels in the serum in healthy individuals [54] and can increase 1000-fold during active infection. During infection in an animal model, PCT is released from many cell types distributed throughout the body [55] and is induced by interleukin-1β, tissue necrosis factor (TNF)-α, IL-6, and lipopolysaccharides and can be attenuated by interferon-γ that is elevated during viral infections [56]. These and other observations have led to the extensive evaluation of PCT as a marker of sepsis and bloodstream infection.

While interpretations of many biomarkers suffer from elevations in conditions other than bacterial infection, PCT has shown promise in improved specificity in bacterial infections. Early studies showed that PCT showed differences in infectious versus noninfectious, inflammatory conditions [57–59]. It has been shown to be able to differentiate between patients with confirmed bacterial *versus* viral infections with high sensitivity (95%) [60]. It has also been used to evaluate secondary bacterial superinfection in patients admitted with influenza [61, 62]. PCT had high sensitivity to exclude bacteremia in urosepsis [63] and community acquired pneumonia [64]. Additionally, there have been studies showing that bacteremia is highly unlikely when PCT levels are below the threshold 0.1 ng/mL [65].

There have been several meta-analyses evaluating PCT as a diagnostic marker in sepsis [33, 66–68]. While the earlier meta-analyses had conflicting results and were limited by populations studied and sepsis definitions, the most recent meta-analysis [68] evaluating 30 studies with 3244 patients yielded a sensitivity of 77% (95% confidence interval (CI): 72–81%) and specificity of 79% (CI: 74–84%) indicating that it was a useful biomarker for diagnosis of early sepsis, but could not be used in isolation and must be interpreted in context of patient presentation.

Several studies have evaluated PCT for diagnosis of sepsis in an emergency department (ED) setting. A meta-analysis published in 2007 including 17 studies found a sensitivity of 76% and specificity of 70% for the detection of sepsis; however, these studies were heterogeneous in the prevalence of sepsis and PCT cutoffs used for diagnosis [69]. A more recent study evaluated 336 adult emergency room patients of which 60% had definite infection; PCT levels were higher in septicemia (median PCT 2.3 versus 0.2 ng/mL) and concentrations increased with likelihood of infection and sepsis severity [70]. PCT best predicted septicemia when compared with IL-6 and CRP with 73% sensitivity and 70% specificity for bacteremia with a cutoff of 0.5 ng/mL.

PCT levels have been found to differ between medical and surgical patients with septic shock with higher baseline levels in surgical patients proposed to be due to transient bacteremia, endotoxin release, or ischemia, and a higher threshold value (9.7 ng/mL) had higher sensitivity (92%) for surgical patients [71]. PCT levels have also found to differ for neonatal patients with sepsis with a meta-analysis showing that neonates with sepsis and meningitis sensitivity ranged from 81 to 100% [72].

PCT does not appear to be affected by neutrophil count and has been evaluated in patients presenting with neutropenic fever. A recent meta-analysis of 30 studies evaluating PCT in neutropenic patients found PCT to be helpful, but not diagnostic for bacteremia due to lack of standard definitions, heterogeneity of study populations, and small number of patients included in some of the studies [73]. Knowing that PCT levels increase with severity of infection and over time, serial levels may be indicated in this population. It should be noted that PCT remains unaffected by corticosteroids when compared with CRP [74].

Data are not sufficient to make determinations of PCT use in fungemia. PCT levels in candidemia do not appear to show the same level of elevation as in bacteremia; one retrospective analysis of bacteremia and candidemia in nonneutropenic patients showed a significantly lower PCT level in candidemic patients [75]. However, most of the studies evaluating PCT in invasive fungal infections are limited by small case counts; a recent meta-analysis including 8 studies with 474 episodes of suspected infection (155 confirmed or probable invasive fungal infections) showed a pooled sensitivity of 0.82 (95% CI, 0.48–0.95) and specificity of 0.80 (95% CI, 0.60–0.91) [76]. They noted the negative likelihood ratio could not be used to safely exclude systemic fungal infection. It should be noted that the studies included had a wide range of PCT cutoffs (0.3–5.5 ng/mL).

PCT has been investigated in several studies in India, though most have focused on case reports or series looking at specific diagnoses such as scrub typhus [77], septic arthritis and osteomyelitis [78], H1N1 [79], pancreatitis [80, 81], pyelonephritis [82], and meningitis [83].

One Indian study looked at PCT with a semiquantitative PCT test as well as eubacterial PCR in comparison with blood cultures [84]. Ninety patients (60 septic patients compared with 30 nonseptic patients) were evaluated; compared with blood cultures, the sensitivity, specificity, and positive and negative predictive values for PCT were 100%, 62%, 57%, and 100%, respectively. The authors concluded that PCT may be useful as a rapid test for detecting septicemia but compared with blood cultures lacked specificity which may be in part to the high cutoff value of 2 ng/mL that was used in this study.

A more recent prospective study in India conducted from 2006 to 2008 evaluated 100 patients and found a sensitivity of 94% with a significant association with Sequential Organ Failure Assessment scores, but no significance for severity of sepsis or mortality [85]. Another study conducted in an Indian ICU setting evaluated 40 patients found that patients with PCT $\geq$ 2 ng/mL had statistically significant correlation with the presence of sepsis ($P < 0.0001$) with a moderate sensitivity (86%) and high specificity (95%) [86]. It should be noted that a 2 ng/mL threshold is higher than many studies (0.1–2 ng/mL). PCT was evaluated as a biomarker in neonatal sepsis in 118 neonates with early onset sepsis compared with 61 normal samples [45]. There were significantly higher levels of PCT ($1.500 \pm 0.2400\ \mu$g/L) in neonates with sepsis. Obviously, there is great opportunity to study PCT in India.

4. PCT for Antimicrobial Stewardship

Due to its ability to help differentiate between viral and bacterial infections, PCT has been evaluated for its ability to guide decisions for appropriate antibiotic therapy. India has one of the highest rates of infectious diseases and has alarmingly high rates of resistant bacteria, making utilization of diagnostics that help indicate when unnecessary antibiotics can be avoided a prime goal [87, 88]. Initially there were two small, single center studies investigating the use of PCT in an antibiotic management algorithm for septic patients. The first evaluated serial PCT measurements in 39 patients compared with 40 controls in an ICU setting and found a 4-day reduction in the duration of antibiotic therapy ($P = 0.003$) and a smaller overall antibiotic exposure ($P = 0.0002$), 2-day shorter ICU stay ($P = 0.03$) without a difference in 28-day mortality, clinical cure, or relapse [89]. The second evaluated surgical ICU patients and found a decrease in antibiotic exposure days (5.9 ± 1.7 versus 7.9 ± 0.5 days, $P < 0.001$) and decrease in length of stay without negative effects on outcomes [90]. The PRORATA trial, a multicenter, prospective, open-label, and randomized control trial including 621 patients in 8 ICUs in 6 hospitals followed these smaller studies and found a 23% reduction in antibiotic usage at day 28 [91]. Importantly, PCT guided deescalation in antibiotics was noninferior to standard of care with a 10% noninferior mortality difference assumed. They cautioned not to extrapolate results to surgical ICU patients as they comprised only 10% of the population and concluded that PCT-based algorithms are likely to have the greatest benefit at aiding discontinuation of antibiotics rather than withholding them from critically ill patients especially given that no ideal threshold for starting or withholding antibiotics in critically ill patients has been established. PCT measurements have also been found to be statistically significantly higher in patients with true bacteremia when compared to patients deemed to have contaminants with coagulase negative staphylococci [92] which certainly have implications for decreasing inappropriate antibiotic use. There have been numerous other studies evaluating deescalation of antibiotics in a variety of clinical syndromes, including respiratory disease [93].

There have been several meta-analyses of PCT algorithms that evaluated antibiotic use. Three evaluated a variety of infections [94–96]. The first included 14 randomized controlled trials (RCTs) ($N = 4467$ patients) that investigated PCT algorithms for antibiotic treatment decisions in adult patients with respiratory tract infections and sepsis from primary care, ED, and ICU settings. There was no difference in mortality in any setting. In the primary care setting, 2 studies found 74–42% reduction in antibiotic prescription and a 13–0% [97, 98]; in the ED 6 trials found reduction in antibiotic prescription from 47 to 15% and duration from +8% to −55% [99–101]; in the ICU setting 6 trials showed reduction in duration from 37 to 20% [89, 102]. The second review [95] evaluated 6 published RCTs comparing PCT-guided antimicrobial therapy to usual care in ICU patients; PCT guidance was associated with significantly reduced antimicrobial exposure (effect sizes, 20%–38%). The third [96] included 7 studies with 1458 patients (4 with respiratory infections, 2 with septic patients, and 1 with surgical ICU patients) and found that PCT-guided therapy was associated with reduction in antibiotic use at inclusion (4 studies, pooled OR 0.506, 95% CI 0.290–0.882, $P = 0.016$), duration of antibiotic therapy (6 studies, weighted mean difference (WMD) 2.785, 95% CI 1.225–4.345, $P = 0.000$), and total antibiotic exposure days/1,000 days (4 studies, pooled RR 1.664, 95% CI 1.155–2.172, $P = 0.000$) without differences in length of total hospital stay or mortality.

Four meta-analyses [103–106] evaluated PCT-guided treatment on antibiotic usage in ICU patients. Heyland et al. found that PCT-guided treatment was associated with a significant reduction in antibiotic use (WMD −2.14 days, 95% CI: −2.51 to −1.78, $P < 0.00001$) [103]. Another meta-analysis [104] included 7 RCTs with 1131 ICU patients and found that use of PCT-guided strategies decreased the duration of antibiotic therapy for the first episode of infection (WMD −2.36 days, 95% CI: −3.11 to −1.61) and the total duration of antibiotic treatment by 4 days (WMD −4.19 days, 95% CI: −4.98 to −3.39) without difference in 28-day mortality or relapse infection rate. Another analysis [105] incorporated 7 studies, 6 of which were included in the previous meta-analysis [104] and found similar decreases for antibiotic use for first infection episode (pooled WMD = −3.15 days, 95% CI: −4.36 to −1.95, $P < 0.001$) and no difference in mortality. The most recent meta-analysis by Prkno et al. [106] was the first to analyze PCT-guided treatment effects on antibiotic use and clinical outcomes in ICU patients with severe sepsis and septic shock. In that analysis 7 studies consisting of 1075 patients observed that while mortality and length of stay were no different between PCT-guided treatment and standard of care, there was a statistically significant decrease in duration of antibiotic use in the PCT-guided approach (hazard ratio: 1.27, 95% CI: 1.01; 1.53). They comment that using a PCT-guided approach for treatment of severe sepsis reduces antibiotic exposure without an obvious difference in mortality, though more research is needed to further define the PCT algorithms used in different patient populations as different cutoff values were used for different patient populations with differences noted between medical and surgical patients with severe sepsis.

Kaur et al. published a review in 2013 evaluating PCT in an effort to reduce inappropriate antibiotics in an Indian

emergency setting [107], but no studies evaluating PCT for antibiotic stewardship in India have been published to date.

5. PCT Assays, Cost, and Implementation in India

Currently there are several PCT assays that have been developed and compared in the literature. The main manufacturer of PCT assays is BRAHMS and include the Kryptor, VIDAS, PCT-Q, and PCT LIA assays [108].

PCT sensitive Kryptor assay provides a sensitive, functional assay sensitivity of 0.06 ng/mL with results available in 19 minutes using 20–50 mL of plasma or serum [109]. The BRAHMS PCT LIA is a manual PCT assay that can test plasma or serum using a luminometer and has a detection limit of ~0.3 to 0.5 ng/mL with results available after 1 hour incubation time [110]. The LIASON BRAHMS PCT is a fully automated random access analyser that uses two-site immunoluminometric assay with functional sensitivity of 0.3 ng/mL and can have results within 30 minutes [108]. VIDAS BRAHMS PCT is an enzyme-linked fluorescent immunoassay providing quantitative PCT measurements with a functional detection limit of 0.09 ng/mL [108]. The BRAHMS PCT-Q is a manual immunochromatographic test for the semiquantitative detection of PCT after incubation of 30 minutes and can distinguish ranges above 0.5 ng/mL [108]. The PCT-Q is marketed as a point-of-care testing kit. Results are indicated by four different shades of red, corresponding to different PCT ranges, indicating the possibility and severity of sepsis [111]. Although the kit is designed to require no specialized training, the semiquantitative nature requires interpretation by the operator, and user difficulties in interpreting results have been reported, and its results only showed moderate agreement compared with the Kryptor assay when used in the clinical setting [111]. There are several other PCT platforms available from BRAHMS.

Schuetz et al. reported that the Kryptor and VIDAS systems could be used interchangeably [112], and Steinbach et al. found agreement between the Kryptor and PCT-Q systems for ranges of PCT that were common to both systems [113]; PCT-Q assay has the disadvantage of being able to discriminate values <0.3 ng/mL. The fully automated LIASON system has been found to have good correlation with the previous PCT-Q assay as well [114].

While purchasing a PCT platform can be expensive, there have been several analyses to indicate that PCT-based algorithms might be cost-effective in different patient populations and illnesses [103]. Heyland et al. performed an economic analysis of PCT-based algorithms compared with standard of care in a meta-analysis [103] including five studies, four of which have been referenced earlier in this paper [89–91, 102]. As the results of the analysis demonstrated no difference in mortality, length of stay, or recurrent infection, cost analysis in Canadian dollars evaluated acquisition costs of antibiotics, administration costs of intravenous antibiotics, and PCT testing costs, including assay material, reagents, technician time, purchase, maintenance of a bench top analyzer, and overhead. They utilized three cost scenarios and found an average cost savings per treatment episode of Can$470.62 in 2011. This number increased to over Can$1100 cost saving per episode using more expensive antibiotics but showed an increase in cost over standard therapy by Can$193.64 per patient using the least expensive antibiotic scenario and most frequent PCT testing algorithm. Total cost savings depend on a variety of factors including local costs of the PCT assay, the frequency of PCT measurement, and the cost and duration of the antibiotics used.

It is difficult to translate the above data to India where antibiotic use, availability, and ease of implementing a test are drastically different. As cost changes over time, it is always difficult to use historical studies such as Heyland's which was published in 2011. But as PCT testing becomes more mainstream, testing cost will decrease while still providing opportunity to decrease antibiotic use. As semiquantitative testing, such as the PCT-Q test, is the least expensive and the easiest to implement and provides point-of-care results, this may be the platform of interest until other options are available. The area of cost-effectiveness of PCT testing in sepsis in India is an opportunity for further research.

6. sTREM-1, IL-6, IL-8, IL-27

While PCT has shown the most promise and has been the most studied of biomarkers for sepsis and bloodstream infections to date, there are hosts of other biomarkers that have been evaluated with a recent review indicating at least 180 that have been researched [16]. Triggering receptor expressed on myeloid cells-1 (TREM-1) was reported to be upregulated in various inflammatory diseases as well as in sepsis; TREM-1 expression is associated with elevations in soluble TREM-1 (sTREM-1). Studies have shown that the expression of TREM-1 is elevated *in vitro* in the presence of bacteria or fungi as well as peritoneal fluid and tissue from infected patients [115, 116] but remains at normal levels in noninfectious inflammatory conditions and may be a therapeutic target for sepsis [117].

Some studies have shown sTREM-1 to be superior to CRP and PCT [118] but other studies have shown that sTREM-1 has poor discriminatory power compared with routinely available parameters [119]. A recent meta-analysis found that the sensitivity of sTREM-1 for the diagnosis of bacterial infection was 82% and that the specificity was 86% [120].

While IL-6 and IL-8 levels have been shown to be elevated in sepsis and associated with severity and outcome [121], they have not been found to be superior to PCT as biomarkers [58, 70]. These cytokines have been found to be elevated in neutropenic fever [38] and neonatal sepsis [122] but have been less useful in the adult population [123].

Wong and colleagues looked at genome-wide transcriptional profile differences in leukocytes between infected and noninfected pediatric ICU patients and found 221 differentially expressed probes [124]. Individual patient mosaics were assigned to either noninfectious illness or sepsis classes thereby achieving 90% specificity and 94% PPV. Interleukin-27 (IL-27) presented the highest predictive power. The same group subsequently validated their findings by measuring serum levels of IL-27 in a separate study and found serum IL-27 concentrations were significantly higher in patients with

sepsis in comparison with noninfected patients yielding 92% specificity and 91% PPV for bacterial infection in critically ill children. There are many more candidate biomarkers that have been developed that require more investigation prior to use, including markers of endothelial cell activation [125].

Data in the Indian population on these biomarkers are lacking as a whole. Sugitharini also looked at a variety of mediators in neonatal sepsis [45] including granule-associated mediators (neutrophils elastase (NE), myeloperoxidase (MPO), and nitric oxide (NO)), proinflammatory cytokines (TNFα, IL-1β, and IL-6), anti-inflammatory cytokines (IL-10 and IL-13), chemokines (IL-8 and monocyte chemotactic protein (MCP-1) and novel cytokines). They found significantly higher levels of NE, NO, TNFα, IL-1β, IL-6, and IL-8 in neonates with early onset sepsis compared with controls. The levels of MPO were downregulated, and there was no change in IL-13. The presence of 17 inflammatory proteins including IL-16, TNFα, TNFβ, and MCP-1 were upregulated in neonatal sepsis. This study evaluated a number of biomarkers that are dysregulated during sepsis in a neonatal population suggesting that many of the ideas about biomarkers in studies in Europe and in adult patients may be similar after more study is done.

7. Future Directions

The many biomarkers that are under investigation for sepsis diagnosis and prognosis have been well documented, but no one test is sufficient to exclude bloodstream infection. There has been hope that a combination of biomarkers could create a useful algorithm with adequate sensitivity and specificity to aid in diagnosis.

Initial studies attempted to incorporate PCT into decision models and found that CRP improved model fit and created a resulting score that was more accurate than physician judgment of SIRS alone [126]. Utilizing data from the expanding research on biomarkers, an observational study evaluated 17 immune mediators and employed a combined cytokine score with IL-6, IL-8, and IL-10 and showed it was useful predictor of outcome [127]. A study of 151 patients (96 with bacterial infections) were evaluated with 6 biomarkers including sTREM-1, CRP, and PCT and found that a combination of the markers showed improved diagnostic ability compared with any single maker [128]. Another approach that created a bioscore using PCT, sTREM-1, and CD64 index in 300 consecutive patients and subsequently externally validated the score in an independent prospective cohort of 79 patients found each biomarker to be independent predictors of infection but the performance of the bioscore to be superior to each individual biomarker and significantly elevated ($P < 0.001$) in patients with sepsis compared to noninfected patients [129].

Combination of biomarkers has also been evaluated in a pediatric sepsis model [130]. After a genome-wide expression profiling, a risk stratification tool investigated 12 markers and employed 5 biomarkers in the analysis. The PERSEVERE model was found to have sensitivity for mortality of 93% (79–98), specificity 74% (69–79), PPV 32% (24–41), and NPV 99% (96–100).

Combination models have also been evaluated in neonatal sepsis evaluating four tests (microerythocyte sedimentation rate, immature to total neutrophil count, morphological changes in neutrophils, and CRP) and found the role of these tests in early diagnosis of neonatal sepsis were statistically significant ($P < 0.05$) with a combination of three or all of these four tests was highly specific (95–100%) [131].

In order to advance the field of biomarker research in India, well-designed studies are necessary to evaluate threshold values for the diagnosis and deescalation of antibiotics in Indian patients with sepsis. Additionally, more study of specific subgroups, including pediatric versus adult patients, varying severity of sepsis, medical versus surgical patients, and other populations with specific syndromes is needed in general, and in India, in specific. Further study of investigational biomarkers that may hold promise for evaluating sepsis either as an individual test or in conjunction with other tests to improve sensitivity and specificity needs to be investigated for their potential use in India.

8. Summary

Sepsis continues to be a significant cause of morbidity and mortality despite advances in therapeutics and diagnostics. Biomarkers for sepsis, and by extension bloodstream infections, hold much promise for increasing the rapidity with which sepsis is diagnosed and for risk stratification for prognostication. Despite extensive research, no single biomarker can yet serve as the lone diagnostic parameter. PCT remains the most researched and utilized biomarker for sepsis. While cost effectiveness analyses have been done on PCT in acute respiratory infections, there is still a need for robust cost effective analyses in sepsis [132] which will be of keen interest in India to determine potential rational implementation strategies. While there are publications that come out of India evaluating PCT and other biomarkers in sepsis, the level of evidence is still not such to make definitive recommendations for use. PCT may be an effective tool for utilization in an algorithm for diagnosing sepsis and lessen dependence on microbiology resources that can vary in India. Data about biomarkers and PCT in sepsis are gradually increasing and will help provide informed next steps for research in India.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Vancomycin-Resistant *Enterococcus faecium* Bacteremia in a Tertiary Care Hospital: Epidemiology, Antimicrobial Susceptibility, and Outcome

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Vancomycin-resistant *Enterococcus faecium* (VREF) has emerged as a relevant multidrug-resistant pathogen and potentially lethal etiology of health care associated infections worldwide. The objective of this retrospective cohort study was to assess factors associated with mortality in patients with VREF bacteremia in a major tertiary referral hospital in Southern Brazil. All documented cases of bacteremia identified between May 2010 and July 2012 were evaluated. Cox regression was performed to determine whether the characteristics related to the host or antimicrobial treatment were associated with the all-cause 30-day mortality. In total, 35 patients with documented VREF bacteremia were identified during the study period. The median APACHE-II score of the study population was 26 (interquartile range: 10). The overall 30-day mortality was 65.7%. All VREF isolates were sensitive to linezolid, daptomycin, and quinupristin-dalfopristin. Linezolid was the only antimicrobial agent with *in vitro* activity against VREF that was administered to the cohort. After multivariate analysis, linezolid treatment (HR, 0.08; 95% CI, 0.02–0.27) and presence of acute kidney injury at the onset of bacteremia (HR, 4.01; 95% CI, 1.62–9.94) were independently associated with mortality. Presentation with acute kidney injury and lack of treatment with an effective antibiotic poses risk for mortality in patients with VREF bacteremia.

1. Introduction

Vancomycin-resistant *Enterococcus faecium* (VREF) is currently one of the most important etiologies of nosocomial infections worldwide, mainly due to its typical profile of multidrug resistance and tendency to cause severe infections in critically ill patients [1, 2]. Risk factors for developing a nosocomial VREF infection include prolonged hospitalization; hospitalization in long-term facilities, surgical units, or intensive care units; multiple courses of antibiotics; solid organ and hematopoietic stem cell transplantation; and presence of comorbidities such as diabetes, renal failure, or hemodialysis [3–6]. In the continuum of VREF infections, bacteremia is of special interest, given that overall mortality rates may reach values higher than 60% with an attributable mortality of around 40% [7–12]. Unfortunately, few data are available concerning factors associated with mortality in the context of VREF bacteremia in different institutions. Therefore, we conducted a study with the aim of assessing factors associated with mortality in patients with VREF bacteremia in the current practice of a tertiary referral hospital.

2. Methods

2.1. Study Design, Patients, and Settings. A retrospective cohort study was performed with all cases of documented VREF bacteremia identified between May 2010 and July 2012. The present study was conducted at Hospital de Clínicas de Porto Alegre (HCPA), a major tertiary referral hospital in Southern Brazil. The patients were identified by retrieval from the computerized database established by the Infection Control Center of HCPA. Bacteremia by VREF was defined as 2 positive results of 2 independent blood cultures from a patient with fever (body temperature ≥38°C). Blood isolates

were identified according to standard techniques and Vitek2 (bioMérieux) [13]. VREF was defined as an *Enterococcus faecium* isolate with an MIC of vancomycin ≥32 μg/mL by the Etest (bioMérieux) according to the standards of the CLSI. The analyses of clinical features, antibiotic susceptibility tests, and outcomes were focused on those patients with VREF bacteremia. Medical records of the patients who had VREF bacteremia between May 2010 and July 2012 were reviewed. Patients who had ever developed VREF bacteremia before the study period were excluded. If patients developed several episodes of VREF bacteremia during the study period, only the first episode was investigated.

2.2. Variables. Variables retrieved from a standardized case report form included demographics, underlying comorbidities, APACHE II score (Acute Physiology and Chronic Health Evaluation) at the first 24 hours following clinical signs of bacteremia, initial plasma C-reactive protein, initial serum albumin, presence of acute kidney injury (defined as decreases in glomerular filtration rate >50% or an increase in serum creatinine to ≥1.5 times baseline), and whether the infection was acquired in ICU or clinical ward. Data regarding antimicrobial therapy administered (e.g., type of antibiotic, time to antibiotic, and duration of treatment) were also analyzed. The main outcome of this study was all-cause mortality within 30 days from VREF bacteremia.

2.3. Antibiotic Susceptibility Test. MICs for daptomycin, linezolid, and quinupristin-dalfopristin were determined by the Etest (bioMérieux), according to the manufacturer's guidelines (AB Biodisk). Daptomycin, quinupristin-dalfopristin, and linezolid resistance was defined as an isolate with an MIC greater than 4 μg/mL, 4 μg/mL, and 8 μg/mL, respectively [14, 15]. A suspension of each isolate in Mueller-Hinton broth, adjusted to the density of a 0.5 McFarland standard, was swabbed in three directions to ensure uniform growth onto Mueller-Hinton agar plates. The MIC was read where inhibition of growth intersected the *E*-test strip. When small colonies grew within the zone of inhibition or a haze of growth occurred around MIC endpoints, the highest MIC intersection was recorded.

2.4. Statistical Analysis. A Cox proportional hazards regression was performed to determine risk factors for 30-day mortality in patients with VREF bacteremia. All variables that had a P value <0.10 in a univariate analysis were included. In the multivariate model, independent variables were eliminated from the highest to the lowest P value but remained in the model if the P value was less than 0.05. Hazard ratios were estimated along with 95% confidence intervals. Kaplan-Meier curves were used to calculate the time-dependent occurrence of death; the log-rank test was used for comparisons between groups. The software used for the statistical analysis was STATA version 12 (StataCorp LP, USA).

2.5. Ethics. The study was approved by the institutional review board of Hospital de Clínicas de Porto Alegre.

Table 1: Clinical characteristics of 35 patients with bloodstream infection by vancomycin-resistant *Enterococcus faecium*.

Age, years, median (IQR)	46.0 (32.0)
Female sex	14 (40.0)
Type of underlying disease	
Hematologic malignancy	9 (25.7)
Solid malignancy	7 (20.0)
Cirrhosis	4 (11.4)
Diabetes mellitus	3 (8.5)
Connective tissue disease	2 (5.7)
Chronic obstructive pulmonary disease	1 (2.8)
Others*	9 (25.7)
APACHE II score, median (IQR)	26 (10)
Initial plasma CRP, mg/L, median (IQR)	128.5 (177.0)
Initial serum albumin, g/L, median (IQR)	2.4 (1.0)
Acute kidney injury at the onset of bacteremia	12 (34.2)
ICU-acquired bloodstream infection	22 (62.8)

Data presented as n (%) unless otherwise indicated. IQR: interquartile range (P75–P25); CRP: C-reactive protein; ICU: intensive care unit. *Others include isolated cases of heart failure, abdominal aortic aneurysm, acute mesenteric ischemia, ischemic stroke, spinal cord injury, vesicorectal fistula, necrotizing fasciitis, cytomegalovirus colitis, and spontaneous pneumothorax.

3. Results

In total, 35 patients with VREF bacteremia were evaluated during the study period. As shown in Table 1, the overall mean age of the study cohort was 46 years and 60% were male. Subjects with malignant neoplasm comprised 45.7% of the study population; hematologic malignancies accounted for most cases of cancer. Other important underlying comorbidities found were cirrhosis (11.4%) and diabetes mellitus (8.5%). All cases of VREF bacteremia were acquired after 48 hours of hospitalization (62.8% acquired in the intensive care unit and 37.2% acquired in the clinical ward). The median APACHE II value of all study patients was 26.0.

All VREF isolates had a vancomycin MIC ≥256 μg/mL. The most common antibiotics initially administered to patients were vancomycin (48.5%), meropenem (42.8%), and piperacillin-tazobactam (14.2%). Linezolid was the only antimicrobial agent with *in vitro* activity against VREF that was administered to the cohort; 26 subjects (74.2% of the study population) were treated with linezolid (88.4% were treated via intravenous route; the remainder were treated via enteral route). The median time to linezolid treatment was 3 days (interquartile range [IQR]: 2 days). The median duration of linezolid treatment was 9.5 days (IQR: 7 days). The antibiotic schemes administered to the 9 patients that did not receive linezolid were vancomycin monotherapy (2 cases), cefepime monotherapy (2 cases), imipenem-cilastatin + clindamycin (1 case), meropenem + vancomycin (1 case), meropenem + vancomycin + gentamicin (1 case), piperacillin-tazobactam + vancomycin (1 case), and meropenem + metronidazole (1 case). The main reason for withholding linezolid was the sudden clinical deterioration of patients, in the context of lack of empiric effective antimicrobial treatment against VREF, resulting in death before blood

Table 2: Univariate Cox regression analysis of risk factors for 30-day mortality in patients with vancomycin-resistant *Enterococcus faecium* bacteremia.

Variable	Mortality group (n = 23)	Survival group (n = 12)	HR (95% CI)	P value
Age, years, median (IQR)	49 (35.0)	44 (31.5)	1.01 (0.99–1.03)	0.23
Hematologic malignancy	7 (30.4)	2 (16.6)	1.33 (0.54–3.29)	0.52
Solid malignancy	4 (17.4)	3 (25.0)	0.64 (0.21–1.92)	0.43
Cirrhosis	4 (17.4)	0 (0)	2.72 (0.91–8.15)	0.07
Diabetes mellitus	2 (8.7)	1 (8.3)	1.10 (0.25–4.72)	0.89
Chronic obstructive pulmonar disease	1 (4.3)	0 (0)	3.28 (0.41–25.9)	0.25
APACHE II score, median (IQR)	26 (10)	28 (4)	0.97 (0.91–1.04)	0.52
Initial plasma CRP, mg/L, median (IQR)	121.3 (92.2)	222.2 (321.9)	0.99 (0.99-1.00)	0.21
Initial serum albumin, g/L, median (IQR)	2.3 (1.1)	2.8 (0.8)	0.74 (0.29–1.91)	0.54
Acute kidney injury	11 (47.8)	1 (8.3)	3.65 (1.58–8.41)	0.002
ICU-acquired bacteremia	17 (73.9)	5 (41.6)	1.84 (0.72–4.69)	0.19
Linezolid treatment	15 (65.2)	11 (91.6)	0.09 (0.33–0.29)	<0.001
Linezolid MIC, microgram/mL, mean ± SD	0.95 ± 0.28	0.97 ± 0.22	0.78 (0.20–2.96)	0.72
Time to linezolid treatment, days, median (IQR)	2.5 (1.0)	4.0 (5.0)	0.77 (0.57–1.05)	0.11
Duration of linezolid treatment, days, median (IQR)	9.5 (6)	10.5 (10)	0.90 (0.80–1.02)	0.11

Data presented as n (%) unless otherwise indicated. HR: hazard ratio; 95% CI: 95% confidence interval; IQR: interquartile range (P75–P25); CPR: C-reactive protein; ICU: intensive care unit; MIC: minimum inhibitory concentration; SD: standard deviation.

Table 3: Multivariate Cox regression analysis of factors associated with 30-day mortality in patients with vancomycin-resistant *Enterococcus faecium* bacteremia.

Variable	Adjusted HR	95% CI	P value
	Model I		
Linezolid treatment	0.08	0.02–0.27	<0.001
Acute kidney injury	4.01	1.62–9.94	0.003
	Model II		
Linezolid treatment	0.13	0.03–0.47	0.002
Initial serum creatinine, g/dL	1.58	1.09–2.29	0.01

HR: hazard ratio; 95% CI: 95% confidence interval.

culture results (88.8% of cases). Acute kidney injury occurred at the onset of VREF bacteremia in 12 patients, of which 50% were treated with linezolid. As expected, the median APACHE II score was higher for patients with acute kidney injury in comparison with patients without acute kidney injury (28.1 [IQR: 8] versus 25.5 [IQR: 12]). The overall 30-day cohort mortality was 65.7% (23 patients).

The distribution of specific antibiotic MICs for VREF (Figure 1) shows a favourable *in vitro* susceptibility of all VREF blood isolates to linezolid, daptomycin, and quinupristin-dalfopristin: no case of resistance to these antibiotics was identified.

In the univariate analysis of the factors associated with 30-day mortality (Table 2), treatment with linezolid ($P < 0.001$) was associated with higher survival rates. Presentation with acute kidney injury at the onset of VREF bacteremia was more frequent in nonsurvivors ($P = 0.002$). There was a tendency of association between presence of cirrhosis and the mortality risk ($P = 0.07$). Other variables related to linezolid treatment (e.g., time to antibiotic and duration of treatment) were not associated with the 30-day mortality rate.

After the multivariate Cox proportional hazards model was performed (Table 3, model I), treatment with linezolid

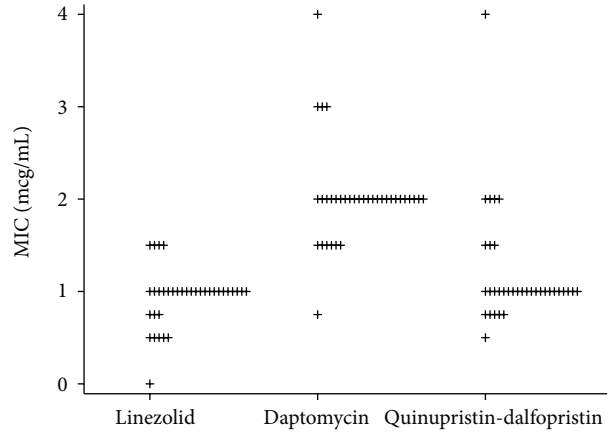

Figure 1: Distribution of specific antibiotic MICs for vancomycin-resistant *Enterococcus faecium* isolates. Note: MIC, minimum inhibitory concentration, microgram/mL.

was independently associated with a higher survival rate (HR, 0.08; 95% CI, 0.02–0.27), while presence of acute kidney injury at the onset of bacteremia constituted an independent

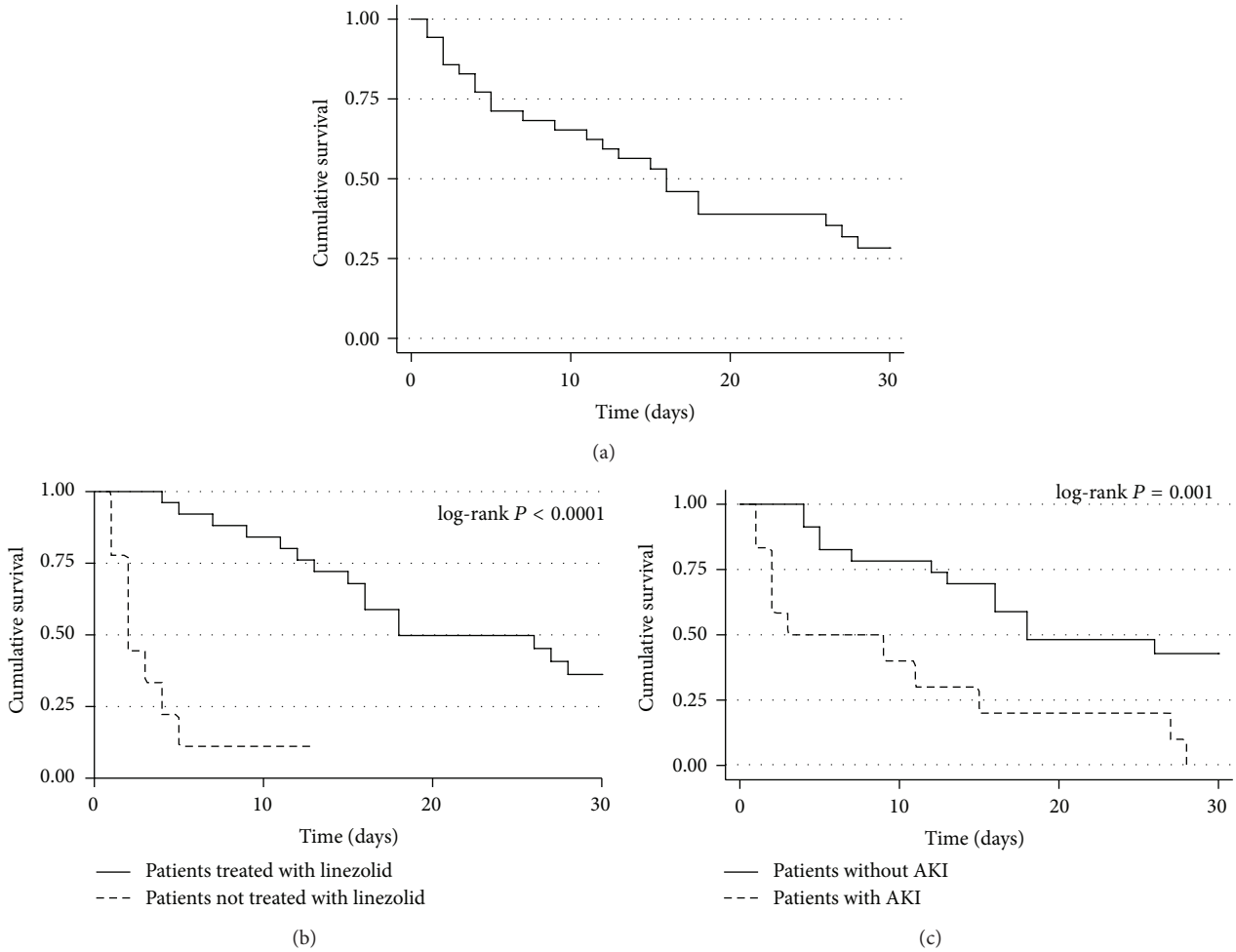

Figure 2: Survival curves of patients with vancomycin-resistant *Enterococcus faecium* (VREF) bacteremia. (a) Survival curve of the entire cohort of patients with VREF bacteremia. (b) Comparison of survival curves of patients treated with linezolid and those treated with other antibiotics without *in vitro* activity against VREF. (c) Comparison of survival curves of patients who presented with acute kidney injury (AKI) at the onset of VREF bacteremia with those who did not present with AKI.

risk factor for 30-day mortality (HR, 4.01; 95% CI, 1.62–9.94). A second multivariate Cox regression model was performed replacing the categorical variable acute kidney injury by the continuous variable initial serum creatinine, while keeping unchanged other variables that reached criteria for entrance in the multivariate analysis (Table 3, model II). This procedure was conducted in order to verify a quantitative relationship between serum creatinine levels and the mortality risk. Each increase of 1.0 mg/dL in the initial serum creatinine level raised the risk of 30-day mortality by 58% ($P = 0.01$).

The survival curves of the entire cohort according to linezolid treatment and the presence of acute kidney injury at the onset of bacteremia are shown in Figure 2.

4. Discussion

Enterococcus is the third most common cause of nosocomial bloodstream infection. VRE is an important problem in Europe, USA, and Latin America and has been isolated in many other countries. Infections due to VRE have been shown to be associated with significant in-hospital mortality and morbidity. Although VRE was first isolated in 1986, the percentage of nosocomial enterococci with vancomycin resistance increased 20-fold in the last 20 years especially among patients in intensive care units, with reported rates of vancomycin resistance varying internationally from 0% to 35% [16, 17]. Despite the fact that 85–90% of clinical isolates of enterococci are *E. faecalis*, most VRE are *E. faecium* [2]. Similarly, in our institution, the vast majority of VRE bacteremia cases are caused by *E. faecium*. The present study showed a significant incidence of VREF bacteremia among patients with solid and hematologic malignancies as previously described in other studies [9, 10, 18, 19]. Moreover, VREF bacteremia compromised mostly ICU patients with high APACHE II scores, a fact that underscores the relevance of VREF infections in critically ill patients. Although resistance to linezolid, daptomycin, and quinupristin-dalfopristin has been reported in VREF isolates [20, 21], our VREF isolates remained highly susceptible to these antibiotics.

Presentation with acute kidney injury at the onset of VREF bacteremia was more frequent in nonsurvivors. This association has been previously suggested only in studies that have been limited by small numbers of patients and a failure to perform multivariate analysis [19, 22]. Additionally, previous reports estimated renal function solely from blood urea nitrogen and creatinine levels, whereas we used the creatinine clearance, a more physiological estimate of renal function.

Our overall 30-day cohort mortality of 66% was comparable with published data, which range from 17% to 100% [23]. The attributable mortality could not be assessed considering that our study did not perform a case control matched analysis with patients without VREF bacteremia. The survival rate was mainly a result of specific therapy against *E. faecium*. Even with the previous data showing a low bactericidal activity of the oxazolidinone antimicrobial agent against VRE [24, 25], in the present study, linezolid was proved to be an effective therapy against VREF bacteremia in a setting of high prevalence of immunocompromised hosts. Interestingly, time to antibiotic use and duration of antibiotic therapy did not play an important role in the main outcome of our patients.

The retrospective analysis of a relative small cohort of patients is the major limitation of our study considering that we cannot be certain that we have identified all potential confounding factors.

5. Conclusions

In summary, our data provide further evidence that VREF is an important cause of mortality in critically ill patients especially with solid and hematological malignancies and renal failure in the ICU setting of a tertiary care institution in Latin America. Despite broad susceptibility to the alternative antimicrobial agents including linezolid and daptomycin against VREF, therapy with ineffective agents for VREF blood stream infections contributed to the poor outcome of the patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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