The Molecular Detection of Class B and Class D Carbapenemases in Clinical Strains of Acinetobacter calcoaceticus-baumannii Complex: The High Burden of Antibiotic Resistance and the Co-Existence of Carbapenemase Genes

Hasan Ejaz 1,*, Muhammad Usman Qamar 2,*, Kashaf Junaid 1, Sonia Younas 3, Zeeshan Taj 2, Syed Nasir Abbas Bukhari 4, Abualgasim E. Abdalla 1, Khalid O. A. Abosalif 1, Naveed Ahmad 5,*, Zikria Saleem 6 and Eman H. M. Salem 7,8

1 Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Jouf University, Sakaka 72388, Saudi Arabia
2 Department of Microbiology, Faculty of Life Sciences, Government College University Faisalabad, Faisalabad 38000, Pakistan
3 HKU-Pasteur Research Pole, School of Public Health, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong, China
4 Department of Pharmaceutical Chemistry, College of Pharmacy, Jouf University, Sakaka 72388, Saudi Arabia
5 Department of Pharmaceutics, College of Pharmacy, Jouf University, Sakaka 72388, Saudi Arabia
6 Department of Pharmacy Practice, Faculty of Pharmacy, Bahauddin Zakariya University, Multan 60000, Pakistan
7 Department of Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University, Shebin El-Koom 32511, Egypt
8 Department of Microbiology, King AbdulAziz Specialist Hospital, Sakaka 72341, Saudi Arabia
* Correspondence: hetariq@ju.edu.sa (H.E.); musmanqamar@gcuf.edu.pk (M.U.Q.)

Abstract: The emergence of carbapenem-resistant Acinetobacter calcoaceticus-baumannii complex (CRACB) in clinical environments is a significant global concern. These critical pathogens have shown resistance to a broad spectrum of antibacterial drugs, including carbapenems, mostly due to the acquisition of various β-lactamase genes. Clinical samples (n = 1985) were collected aseptically from multiple sources and grown on blood and MacConkey agar. Isolates and antimicrobial susceptibility were confirmed with the VITEK-2 system. The modified Hodge test confirmed the CRACB phenotype, and specific PCR primers were used for the molecular identification of bla<sub>CRE</sub> and bla<sub>NDM</sub> genes. Of the 1985 samples, 1250 (62.9%) were culture-positive and 200 (43.9%) were CRACB isolates. Of these isolates, 35.4% were recovered from pus samples and 23.5% from tracheal secretions obtained from patients in intensive care units (49.3%) and medical wards (20.2%). An antibiogram indicated that 100% of the CRACB isolates were resistant to β-lactam antibiotics and β-lactamase; carbapenemase; β-lactams; bla<sub>CRE</sub>; and bla<sub>NDM</sub>; bla<sub>OXA</sub>; bla<sub>VIM</sub> genes. Of these isolates, 94.5% exhibited bla<sub>OXA-51</sub> gene, while bla<sub>OXA-23</sub> was present in 37%, and bla<sub>NDM</sub> in 14% of the isolates. The bla<sub>OXA-51</sub>, bla<sub>OXA-23</sub>, and bla<sub>OXA-24</sub> genes co-existed in 13 (6.5%) isolates. CRACB isolates with co-existing bla<sub>OXA-23</sub> and bla<sub>OXA-24</sub>, bla<sub>NDM</sub>, bla<sub>OXA-51</sub> and bla<sub>VIM</sub> genes were highly prevalent in clinical samples from Pakistan. CRACB strains were highly critical pathogens and presented resistance to virtually all antibacterial drugs, except tigecycline and colistin.

Keywords: Acinetobacter calcoaceticus-baumannii; β-lactamase; carbapenemase; β-lactams; bla<sub>CRE</sub>; bla<sub>NDM</sub>; bla<sub>OXA</sub>; bla<sub>VIM</sub>
1. Introduction

Carbapenem-resistant *Acinetobacter calcoaceticus-baumannii* complex (CRACB) is a well-known nosocomial pathogen that causes severe public health problems, primarily in low- and middle-income countries (LMICs) [1,2]. Recent data indicate that antimicrobial resistance (AMR) is becoming the leading cause of death; bacterial AMR was associated with 4.95 million deaths worldwide in 2019, of which 1.27 million deaths were caused by AMR, with relatively higher mortality among LMICs due to their fragile healthcare systems [3]. During the COVID-19 rife, excessive antimicrobial use resulted in an increase in AMR, which continues to pose a significant threat to healthcare [4]. *A. baumannii* is an important member of the non-Enterobacterales; this well-known pathogen is widespread and has a remarkable ability to develop AMR and cause persistent infections of hospital origin. It is responsible for high death rates ranging from 18.3% to 88.7%, depending on the source of infection [5]. As a result, the World Health Organization (WHO) has elevated it to the top of the list of critical pathogens. The Center for Disease Control and Prevention (CDC) placed it in the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) pathogens list [6]. Recent US data show that CRACB caused an estimated 700 deaths in 2017, and has contributed USD 281 million to health care costs annually [7].

Although carbapenems are the most powerful agents in the antibiotic armamentarium, the production of carbapenemases by bacteria, such as the CRACB strains that produce New Delhi metallo-β-lactamase (NDM) and oxacillinases (OXA), has immensely endangered the bactericidal properties of these medicines [8,9]. *A. baumannii*, which produces NDM and OXA, is resistant to various antibiotics and is only treated with antibiotics, such as colistin and polymyxin B, which are on the WHO’s critically important list of antimicrobials [10]. NDM was initially isolated from *Escherichia coli* and *Klebsiella pneumoniae* in 2009 from a Swedish patient admitted to a hospital in New Delhi, India [11]. In addition to carbapenem resistance mediated by oxacillinases, carbapenem activity is also impaired by metallo-β-lactamases (MBL), in particular by subclass B1 (NDM, VIM, and IMP) [12].

Depending on the amino acid sequence and substrate specificity, β-lactamases are divided into four Ambler’s classes: class A (penicillinases); class B (metallo-β-lactamases); class C (cephalosporinases); and class D (oxacillinases). The *blaNDM* gene encodes a metallo-β-lactamase in subfamily B1 of class B and *blaOXA*, a class D β-lactamase [13]. Both of these β-lactamases have the highest number of genetic variants out of a total of 7420 β-lactamases (class B: 799; class D: 1142) [14]. Intrinsic *blaOXA-51* is a chimeric determinant of *A. baumannii*, whereas *blaOXA-23* and *blaOXA-24* are acquired from *Acinetobacter radioresistens*. The continued increase in the number of these determinants due to unregulated diffusion is worrisome, as it challenges the effectiveness of antimicrobial agents, especially carbapenems [12]. Therefore, the present study highlights the acquisition, co-existence, and spread of these genetic determinants and challenges to infection control programs in hospitals.

2. Results

2.1. Prevalence of CRACB in the Clinical Setting

Of the 1985 clinical samples, 735 (37%) were bacterial culture-negative, and 1250 (62.9%) were culture-positive. Of the positive cultures, 795 (63.6%) isolates belonged to the Enterobacterales family and were excluded from the study, and the remaining 200 (43.9%) CRACB isolates were processed. Of the CRACB isolates, 79 (35.4%) were recovered from pus samples, 47 (23.5%) from tracheal secretions, 21 (10.5%) from sputum, and 20 (10%) from blood samples. The age range was 1 to 95 years, and the mean age was 43.5 years. Most of the isolates were recovered from males, and the male-to-female ratio was 1.27:1. The clinical samples were mainly collected from intensive care units (ICUs; 76 [38%]), followed by male medical wards (MMWs; 37 [18.5%]), female medical wards (FMWs; 36 [18%]), and critical care units (CCUs; 30 [15%]). There was no statistically significant association between the frequency of CRACB and gender, clinical samples, or ward; however, there was a significant association with age ($p = 0.05$) (Table 1).
Table 1. Clinical characteristics and patient demographics of CRACB and CSACB cases.

| Clinical Characteristic | CRACB (n = 200) | CSACB (n = 255) | p-Value |
|-------------------------|-----------------|-----------------|---------|
| Age (Years)             |                 |                 |         |
| Range                  | 1–95            | 1–87            |         |
| Mean (±standard deviation) | 43.5 ± 17.5    | 46.8 ± 19.2     | 0.05    |
| Gender                  |                 |                 |         |
| Male                    | 112             | 152             | 0.05    |
| Female                  | 88              | 103             |         |
| Male to female ratio    | 1.27: 1         | 1.47:1          |         |
| Clinical Sample         |                 |                 |         |
| Pus                     | 79              | 102             | 0.91    |
| Tracheal secretions     | 47              | 59              | 0.92    |
| Sputum                  | 21              | 33              | 0.92    |
| Blood                   | 20              | 27              | 0.92    |
| CSF                     | 17              | 11              | 0.92    |
| Urine                   | 11              | 16              | 0.92    |
| Bile fluid              | 5               | 7               | 0.92    |
| Hospital Ward           |                 |                 |         |
| ICU                     | 77              | 93              | 0.65    |
| MWW                     | 37              | 46              | 0.89    |
| FMW                     | 36              | 41              | 0.58    |
| CCU                     | 30              | 39              | 0.93    |
| OPD                     | 12              | 21              | 0.36    |
| OT                      | 8               | 15              | 0.34    |

CSF: cerebrospinal fluid; ICU: intensive care unit; MWW: male medical ward; FMW: female medical ward; CCU: critical care unit; OPD: outpatient department; OT: operation theater.

2.2. Minimum Inhibitory Concentration of Antibiotics against CRACB

Antimicrobial susceptibility testing revealed that all (100%) CRACB strains were resistant to all β-lactam antibiotics (cephalosporins and carbapenems) and β-lactamase inhibitors (ticarcillin/clavulanate and piperacillin/tazobactam), 86.5% to quinolone (ciprofloxacin), and 83.5% to aminoglycoside (amikacin) and tetracycline (minocycline), while the most effective antibiotics against CRACB were tigecycline and colistin (Figure 1). The WHO Expert Committee on the Selection and Use of Essential Medicines classified antibiotics into three categories: access, watch, and reserve (AWaRe). In this study, all (100%) CRACB displayed resistance to 8 different AWaRe antibiotics, 157 (78.5%) to 9 AWaRe antibiotics, 147 (73.5%) to 10 AWaRe antibiotics, and 146 (73%) to 11 AWaRe antibiotics (Figure 2).

2.3. Detection of Carbapenemases

Of the 200 CRACB isolates, 178 (89%) were positive for carbapenemase production, as shown by the modified Hodge’s test (Figure 3). The sources of the 178 carbapenemase-producing Acinetobacter calcoaceticus-baumannii complex (ACB) were as follows: 74 (41.5%) were from pus; 42 (23.6%) from tracheal secretions; 18 (10.1%) from sputum; 17 (9.5%) from blood; 15 (8.4%) from CSF; 8 (4.5%) from urine; and 4 (2.2%) from bile fluid.

2.4. Clinical Information on CRACB

We found that CRACB was disseminated in different hospital wards and could be isolated from various clinical sources. CRACB was mainly recovered from pus samples (79; 39.5%) followed by tracheal secretions (47; 23.5%), sputum (21; 10.5%), blood (20; 10%) and CSF (17; 8.5%). Most CRACB isolates from pus samples were detected in ICUs (31; 49.3%) and MWWs (16; 20.2%), while isolates from tracheal secretions were mainly collected from ICUs (19; 40.4%) and FMWs (11; 23.4%) and those from sputum and blood samples were...
obtained from MMWs (7; 33.3%) and ICUs (12; 60%), respectively. However, isolates from CSF and urine samples were mainly collected from FMWs (8; 47%) and MMWs (4; 36.6%), respectively (Table 2).

Figure 1. Antibiotic resistance profile of CRACB isolates. The different classes of antibiotics are TIM: ticarcillin/clavulanate; PRL: piperacillin; TZP: piperacillin/tazobactam; CAZ: ceftazidime; FEP: cefepime; IPM: imipenem; MEM: meropenem; CN: gentamicin; CIP: ciprofloxacin; AK: amikacin; MH: minocycline; TGC: tigecycline; CT: colistin.

Figure 2. CRACB isolates concurrently resistant to the WHO AWaRe group of antibiotics. All the isolates were resistant to eight antibiotics.
Figure 3. Phenotypic carbapenemase confirmation by the modified Hodge test.

Table 2. Dissemination of CRACB from clinical sources and hospital wards.

| Clinical Source | ICU | MMW | FMW | CCU | OPD | OT |
|-----------------|-----|-----|-----|-----|-----|-----|
| Pus (n = 79; 39.5%) | 31 (49.3%) | 16 (20.2%) | 12 (15%) | 12 (15%) | 7 (8.8%) | 1 (7.9%) |
| Tracheal secretions (n = 47; 23.5%) | 19 (40.4%) | 7 (14.8%) | 11 (23.4%) | 8 (17%) | 2 (4.2%) | - |
| Sputum (n = 21; 10.5%) | 6 (28.5%) | 7 (33.3%) | - | 2 (9.5%) | 1 (4.7%) | 5 (23.8%) |
| Blood (n = 20; 10%) | 12 (60%) | - | 1 (5%) | 5 (25%) | - | 2 (10%) |
| CSF (n = 17; 8.5%) | 3 (17.6%) | 3 (17.6%) | 8 (47%) | 2 (11.7%) | 1 (5.8%) | - |
| Urine (n = 11; 5.5%) | 3 (27.2%) | 4 (36.3%) | 2 (18%) | - | 2 (18%) | - |
| Bile fluid (n = 5; 2.5%) | 3 (60%) | 1 (20%) | 1 (20%) | - | - | - |

CSF: cerebrospinal fluid; ICU: intensive care unit; MMW: male medical ward; FMW: female medical ward; CCU: critical care unit; OPD: outpatient department; OT: operation theater.

2.5. Genetic Determinants of Carbapenemase in CRACB

The CRACB isolates (n = 200) underwent further analysis to detect the carbapenem-resistant genes. All the isolates exhibited the intrinsic \( \text{blaOXA-51} \) gene (100%), with high prevalence of \( \text{blaOXA-23} \) (n = 189; 94.5%), followed by \( \text{blaVIM} \) (n = 74; 37%), \( \text{blaNDM} \) (n = 28; 14%), and \( \text{blaOXA-24} \) (n = 13; 6.5%). Furthermore, the \( \text{blaOXA-51}, \text{blaOXA-23}, \text{and blaOXA-24} \) genes coexisted in 13 (6.5%) isolates; \( \text{blaOXA-51}, \text{blaOXA-23}, \text{blaOXA-24}, \text{and blaVIM} \) in 7 (3.5%) isolates; and \( \text{blaOXA-51}, \text{blaOXA-23}, \text{blaVIM}, \text{and blaNDM-1} \) in 3 (1.5%) isolates (Figure 4). The CRACB isolates with co-occurring genes displayed higher resistance than those with only a single carbapenemase gene.
2.5. Genetic Determinants of Carbapenemase in CRACB

The CRACB isolates (n = 200) underwent further analysis to detect the carbapenem-resistant genes. All the isolates exhibited the intrinsic \( \text{bla} \) \( \text{OXA-51} \) gene (100%), with high prevalence of \( \text{bla} \) \( \text{OXA-23} \) (n = 189; 94.5%), followed by \( \text{bla} \) \( \text{VIM} \) (n = 74; 37%), \( \text{bla} \) \( \text{NDM} \) (n = 28; 14%), and \( \text{bla} \) \( \text{OXA-24} \) (n = 13; 6.5%). Furthermore, the \( \text{bla} \) \( \text{OXA-51} \), \( \text{bla} \) \( \text{OXA-23} \), and \( \text{bla} \) \( \text{OXA-24} \) genes coexisted in 13 (6.5%) isolates; \( \text{bla} \) \( \text{OXA-51} \), \( \text{bla} \) \( \text{OXA-23} \), \( \text{bla} \) \( \text{OXA-24} \), and \( \text{bla} \) \( \text{VIM} \) in 7 (3.5%) isolates; and \( \text{bla} \) \( \text{OXA-51} \), \( \text{bla} \) \( \text{OXA-23} \), \( \text{bla} \) \( \text{VIM} \), and \( \text{bla} \) \( \text{NDM-1} \) in 3 (1.5%) isolates (Figure 4). The CRACB isolates with cooccurring genes displayed higher resistance than those with only a single carbapenemase gene.

3. Discussion

CRACB is a serious nosocomial pathogen and is the most common in critical care units around the world. Mortality and morbidity of patients with ACB are extremely high, and numerous cases of bacteremia, peritonitis, pneumonia, and urinary tract infections have been reported. ACB also causes ventilator-associated pneumonia, septicemia, and less common but significant skin, soft tissue, abdominal, and nervous system infections [15]. ACB is typically isolated from tracheal secretions and pus. In this study, 35.4% of ACB isolates were recovered from pus samples and 23.4% from tracheal secretions. Nearly identical findings were obtained in a previous study conducted in Islamabad, Pakistan.
with the highest A. baumannii frequencies of 20% in tracheal secretions and 17% in pus samples [16]. In addition, 27.3% of A. baumannii isolates were obtained from pus samples in a study from Nepal [17]. However, an Indian study found a higher prevalence (64%) of A. baumannii in pus samples [18]. The high prevalence in pus samples might have been due to unhygienic practices and the use of unsterilized instrumentation in hospitals.

One of the most effective groups of medicines in the antimicrobial armamentarium is carbapenems, which are particularly suitable for treating multiple ACB infections. Nevertheless, increased resistance of ACB to carbapenems has been universally reported during the past decade [19]. The high level of resistance of ACB to common antibiotics is a serious concern for clinicians. In our study, 100% of the isolated bacteria were resistant to the utmost widely used β-lactams, such as cephalosporins and carbapenems (imipenem and meropenem). A study from Teheran, Iran, also showed 100% resistance to imipenem and meropenem in intensive care patients [20]. Several studies have reported the high resistance of A. baumannii to β-lactams, including carbapenems, in a variety of clinical settings in Pakistan [10,21–23]. In addition, in Bulgaria, A. baumannii isolates were 98.2% and 100% resistant to imipenem and meropenem, respectively [24]. A recent study of Chinese ICU patients found CRACB with significantly high resistance to tigecycline [25]; non-β-lactams (fluoroquinolones, tetracyclines, polymyxins, and aminoglycosides) were more effective against A. baumannii than β-lactams.

Several factors have contributed to the rise of CRACB in community clinical environments in Pakistan, such as the fragile health system, over-the-counter availability of antibiotics, the absence of microbiological diagnostic laboratories, the misuse and overuse of antibiotics, self-medication, and the socioeconomic status of patients [26,27]. The worldwide occurrence of ACB-resistant strains have further increased the concerns about this pathogen. A. baumannii's high capacity to adapt, as well as its acquisition and transfer of the genetic determinants of antibiotic resistance, has rendered existing therapeutic strategies and the last range of antibiotics ineffective [28]. The acquisition and diffusion of carbapenem-resistance genes in ACB is an important factor responsible for the resurgence of resistance to last-line antibiotics. In this study, 100% of CRACB isolates were positive for blaOXA-51, 94.5% for blaOXA-23, 37% for blavIM, and 14% for blaNDM. A recent study in Pakistan identified 100% of isolates with blaOXA-51 and 97% with blaOXA-23 [29], and another Pakistani study found that the blaNDM and blaOXA-23 genes co-occurred in CRACB clinical isolates [10]. The prevalence of blaOXA-23 was found to be 100% in a study conducted in Central China [30]. In addition, two studies from China reported that 95% and 87% of A. baumannii isolates harbored the blaOXA-23 gene [31,32]. In previous years, fewer occurrences of blaOXA-24 in ACB have been reported in Pakistan. However, this study found that blaOXA-24 has spread, and that it also co-exists with blaOXA-23, blaOXA-51, blavIM, and blaNDM in Pakistan, which implies the possibility of a similar pattern of occurrence of these genes in other less-developed countries. The high number of ACBs presenting multidrug resistance is concerning and indicates the necessity of implementing a comprehensive surveillance program for AMR. The frequent isolation of CRACB from ICUs indicates the importance of strict infection control policies in order to minimize the circulation of ACB within the ICUs and the other wards. In LMICs, patients are often accompanied by several attendants, and entry for these attendants should be restricted, particularly in ICUs. Infection risks can be reduced by frequent handwashing, wearing gloves, using disposable materials, and cleaning floors and nursing stations. The role of clinicians is pivotal in controlling the injudicious use of antibiotics. There is a need to improve the knowledge of young doctors regarding AMR in order to expand their competency in choosing the appropriate antibiotic against various multidrug-resistant bacterial strains [33].

4. Materials and Methods

4.1. Ethical Approval and Study Design

After receiving institutional approval, the study followed the Helsinki Declaration’s ethical principles [34]. In addition, the consent of all participants was obtained by assur-
ing each that the data were used solely for research reasons and that their identity was completely concealed. This study adopted a cross-sectional analysis. A total of 1985 clinical samples from different sources (including pus, tracheal swabs, blood, cerebrospinal fluid [CSF], and urine) were referred to the laboratories of the tertiary care hospitals in Lahore and Faisalabad, Pakistan, for culture and sensitivity tests between November 2020 to May 2021. These samples were selected based on the physician’s clinical information and diagnostic judgment of suspected bacterial infection. The 200 CRACB isolates were further investigated for antibiograms and molecular characterization of antimicrobial resistant genes (ARGs) (Figure 5).

4.2. Case Definition

Clinical isolates were classified as carbapenem-sensitive ACB (CSACB) if susceptible to imipenem or meropenem or as CRACB if resistant or requires greater exposure to either antibiotic. Participants with at least one positive CRACB culture were considered to have CRACB. Any patient who had a positive CSACB culture was considered a CSACB case and excluded from the study.

4.3. Confirmation of the CRACB Phenotypic

Bacterial isolates were sub-cultured on MacConkey agar and blood agar, and the plates were aerobically incubated overnight at 37 °C. Preliminary identification was made based on colony morphology, and the isolates were confirmed using cultural characteristics and GN identification cards in an automatic VITEK 2 compact system (bioMérieux, Marcy-l’Étoile, France).
4.4. The Minimum Inhibitory Concentration of Antibiotics

The AMR of various antibiotics was tested against CRACB in the VITEK 2 system (bioMérieux), and the broth microdilution determined the minimum inhibitory concentrations (MICs) method. The antibiotics tested were piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, meropenem, imipenem, gentamicin, tobramycin, tigecycline, minocycline, ciprofloxacin, and colistin. The MIC was interpreted per Clinical and Laboratory Standards Institute (CLSI) 2022 guidelines [35].

4.5. Phenotypic Confirmation of Carbapenemase

As previously described, the modified Hodge’s test was used to confirm carbapenemase production in ACB. In summary, Hinton agar plates were inoculated with an E. coli strain (ATCC 25922) and a meropenem disk was placed in the middle of each plate. Clinical isolates were streaked from the disc’s edge to the entire plate and incubated overnight at 37 °C [36]. A clover indentation was considered a positive result. The presence of carbapenemases was further confirmed using a more recent modified carbapenem inactivation (mCIM) method and was interpreted in accordance with the CLSI guidelines [35]. The isolates were well-preserved at −82 °C in Luria Bertani broth, which contained 14% glycerol.

4.6. Molecular Identification of Carbapenemase Genes

DNA extraction was carried out by the boiling method prior to amplification. All submitted ARG determinants were augmented by nested PCR using sequence-specific primers [37]. The primers that were used in the study are listed in Table 3. The initiation temperature was 94 °C for 5 min, followed by a denaturation temperature of 94 °C for 30 s. Annealing was carried out at 52 °C for 40 s, followed by a final extension at 72 °C for 6 min [38]. Lastly, the amplified fragments were separated according to the molecular weight/size of the base pair and compared to a DNA scale of 100 bps. Fragments were displayed under UV light using a gel documentation system (Bio-Rad, Watford, UK).

Table 3. The list of primers used in the study.

| Target Gene | Primer Sequence (5′-3′) | Size (bp) |
|-------------|------------------------|----------|
| blaOXA23-F  | GATCGGATTGGAGAACCAGA  | 501      |
| blaOXA23-R  | ATTTCTGACCGCATTTCCAT  |          |
| blaOXA24-F  | GGTTAGTTGGGCCCTTAAA  | 246      |
| blaOXA24-R  | AGTGAGCCGAAAAGGGATT  |          |
| blaOXA51-F  | TAATGCTTGTACGGCTTG  | 353      |
| blaOXA51-R  | TGGATTGCACTTCATTTGG  |          |
| blaVIM-F    | GATGGTGTGTTTGGTCGATAA| 390      |
| blaVIM-R    | CGAATGGCCGCAACCAGA   |          |
| blaNDM-F    | GATTTGGCGATCTGTTTTTC| 699      |
| blaNDM-R    | CGAATGGCTCATCAGTCAT  |          |

4.7. Data Analysis

The IBM SPSS v.26 (IBM, Chicago, United States) and GraphPad Prism 9.0.1 (GraphPad Software, Inc., San Diego, United States) were used for data analysis. The variables were analyzed using descriptive statistics, and a chi-square test was done to calculate p-values. In this study, a p-value less than 0.05 was considered significant.

5. Conclusions

The detection of several CRACB in our study is a substantial public health concern. This study found that this highly critical pathogen was resistant to almost all categories of AWaRe antibiotics, except tigecycline and colistin. The blaOXA-23, blaOXA-24, blaNDM, blaOXA-51 and blaVIM genes were found to co-occur in some CRACB isolates. The spread of
these pathogens nationally and internationally raises serious concerns; therefore, an active and effective national AMR surveillance study should be conducted, and the Ministry of Health of Pakistan should implement the National Action Plan on AMR. The use of collective strategies, such as improving cleaning processes, utilizing aseptic techniques, disposing of old furniture, controlling hospital visitors, and applying stringent policies on antibiotic use, may contribute to the elimination of CRACB.

**Author Contributions:** Conceptualization, H.E., M.U.Q., K.J. and K.O.A.A.; methodology, S.Y., M.U.Q., Z.T., Z.S. and E.H.M.S.; formal analysis, H.E., M.U.Q., K.J., S.N.A.B., N.A., A.E.A. and S.Y.; investigations, H.E., S.Y., K.O.A.A., M.U.Q., S.N.A.B., Z.T., Z.S. and E.H.M.S.; resources, H.E., S.N.A.B., K.O.A.A., N.A. and K.J.; data curation, H.E., M.U.Q. and E.H.M.S.; writing—original draft preparation, S.Y., A.E.A., K.J., E.H.M.S., Z.T. and Z.S.; writing—review & editing K.J., N.A., A.E.A. and E.H.M.S.; supervision, H.E. and S.N.A.B.; project administration, H.E.; funding acquisition; H.E. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was funded by the Deanship of Scientific Research at Jof University under Grant Number (DSR2022-RG-0154).

**Institutional Review Board Statement:** This study was approved by the institutional review board.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding authors.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Wareth, G.; Linde, J.; Nguyen, N.H.; Nguyen, T.N.M.; Sprague, L.D.; Pletz, M.W.; Neubauer, H. WGS-Based Analysis of Carbapenem-Resistant Acinetobacter baumannii in Vietnam and Molecular Characterization of Antimicrobial Determinants and MLST in Southeast Asia. *Antibiotics* 2021, 10, 563. [CrossRef]

2. Sharma, A.; Gaind, R. Development of Loop-Mediated Isothermal Amplification Assay for Detection of Clinically Significant Members of Acinetobacter calcoaceticus-baumannii Complex and Associated Carbapenem Resistance. *Front. Mol. Biosci.* 2021, 8, 659256. [CrossRef]

3. Murray, C.J.L.; Ikuta, K.S.; Sharara, F.; Swetschinski, L.; Aguilar, G.R.; Gray, A.; Han, C.; Bisignano, C.; Rao, P.; Wool, E.; et al. Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. *Lancet* 2022, 399, 629–655. [CrossRef]

4. Segala, F.V.; Bavaro, D.F.; Di Gennaro, F.; Salvati, F.; Marotta, C.; Saracino, A.; Murri, R.; Fantoni, M. Impact of SARS-CoV-2 Epidemic on Antimicrobial Resistance: A Literature Review. *Viruses* 2021, 13, 2110. [CrossRef]

5. Cornejo-Juárez, P.; Cevallos, M.A.; Castro-Jaimes, S.; Castillo-Ramirez, S.; Velázquez-Acosta, C.; Martínez-Oliva, D.; Pérez-Oseguera, A.; Rivera-Buendía, F.; Volkow-Fernández, P. High mortality in an outbreak of multidrug resistant Acinetobacter baumannii infection introduced to an oncological hospital by a patient transferred from a general hospital. *PLoS ONE* 2020, 15, e0234684. [CrossRef]

6. Asokan, G.V.; Ramadhan, T.; Ahmed, E.; Sanad, H. WHO Global Priority Pathogens List: A Bibliometric Analysis of Medline-PubMed for Knowledge Mobilization to Infection Prevention and Control Practices in Bahrain. *Oman Med. J.* 2019, 34, 184–193. [CrossRef]

7. CDC. Antibiotic Resistance Threats in the United States. Available online: https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf (accessed on 10 August 2022).

8. Qamar, M.U.; Lopes, B.S.; Hassan, B.; Khurshid, M.; Shaﬁque, M.; Atif Nisar, M.; Mohsin, M.; Nawaz, Z.; Muzammil, S.; Aslam, B.; et al. The present danger of New Delhi metallo-β-lactamase: A threat to public health. *Future Microbiol.* 2020, 15, 1759–1778. [CrossRef]

9. Mancilla-Rojano, J.; Ochoa, S.A.; Reyes-Grajeda, J.P.; Flores, V.; Medina-Contreras, O.; Espinoza-Mazariiego, K.; Parra-Ortega, L.; Rosa-Zamboni, D.; Castellanos-Cruz, M.D.C.; Arellano-Galindo, J.; et al. Molecular Epidemiology of Acinetobacter calcoaceticus-Acinetobacter baumannii Complex Isolated From Children at the Hospital Infantil de México Federico Gómez. *Front. Microbiol.* 2020, 11, 576673. [CrossRef]

10. Ejaz, H.; Ahmad, M.; Younas, S.; Junaid, K.; Abosalif, K.O.A.; Abdalla, A.E.; Alameen, A.A.M.; Elamir, M.Y.M.; Bukhari, S.N.A.; Ahmad, N.; et al. Molecular Epidemiology of Extensively-Drug Resistant Acinetobacter baumannii Sequence Type 2 Co-Harboring bla (NDM) and bla (OXA) From Clinical Origin. *Infect. Drug Resist.* 2021, 14, 1931–1939. [CrossRef]

11. Yong, D.; Toleman, M.A.; Giske, C.G.; Cho, H.S.; Sundman, K.; Lee, K.; Walsh, T.R. Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. *Antimicrob. Agents Chemother.* 2009, 53, 5046–5054. [CrossRef]

12. Toleman, M.A.; Spencer, J.; Jones, L.; Walsh, T.R. blaNDM-1 is a chimera likely constructed in Acinetobacter baumannii. *Antimicrob. Agents Chemother.* 2012, 56, 2773–2776. [CrossRef][PubMed]
13. Tooke, C.L.; Hinchcliffe, P.; Bragginton, E.C.; Colenso, C.K.; Hirvonen, V.H.A.; Takebayashi, Y.; Spencer, J. β-Lactamases and β-Lactamase Inhibitors in the 21st Century. J. Mol. Biol. 2019, 431, 3472–3500. [CrossRef] [PubMed]

14. Naas, T.; Oueslati, S.; Bonnin, R.A.; Dabos, M.L.; Zavala, A.; Dorlet, L.; Retailleau, P.; Iorga, B.I. Beta-lactamase database (BLDB)—Structure and function. J. Enzym. Inhib. Med. Chem. 2017, 32, 917–919. [CrossRef]

15. Ayobami, O.; Willrich, N.; Harder, T.; Okeke, I.N.; Eckmanns, T.; Markwart, R. The incidence and prevalence of hospital-acquired (carbapenem-resistant) Acinetobacter baumannii in Europe, Eastern Mediterranean and Africa: A systematic review and meta-analysis. Emerg. Microbes Infect. 2019, 8, 1747–1759. [CrossRef]

16. Begum, S.; Hasan, F.; Hussain, S.; Ali Shah, A. Prevalence of multi drug resistant Acinetobacter baumannii in the clinical samples from Tertiary Care Hospital in Islamabad, Pakistan. Pak. J. Med. Sci. 2013, 29, 1253–1258. [CrossRef]

17. Yadav, S.K.; Bhujel, R.; Hamal, P.; Mishra, S.K.; Sharma, S.; Sherchand, J.B. Burden of Multidrug-Resistant Acinetobacter baumannii Infection in Hospitalized Patients in a Tertiary Care Hospital of Nepal. Infect. Drug Resist. 2020, 13, 725–732. [CrossRef]

18. Khamari, B.; Lama, M.; Pachi Pulusu, C.; Biswal, A.P.; Lingamallu, S.M.; Mukiria, B.S.; Sahoo, A.K.; Dash, H.S.N.; Sharda, R.; Kumar, P.; et al. Molecular Analyses of Biofilm-Producing Clinical Acinetobacter baumannii isolates from a South Indian Tertiary Care Hospital. Med. Princ. Pract. 2020, 29, 580–587. [CrossRef]

19. Said, D.; Willrich, N.; Ayobami, O.; Noll, I.; Eckmanns, T.; Markwart, R. The epidemiology of carbapenem resistance in Acinetobacter baumannii complex in Germany (2014–2018): An analysis of data from the national Antimicrobial Resistance Surveillance system. Antimicrob. Resist. Infect. Control 2021, 10, 45. [CrossRef]

20. Alavi-Moghadam, M.; Miri, M.; Mokhtari, M.; Kouchek, M.; Goharani, R.; Sistanzad, M.; Safari, S.; Solouki, M. Incidence of imipenem-resistant Acinetobacter baumannii in a general intensive care unit (ICU). Casp. J. Intern. Med. 2014, 5, 186–187.

21. Hasan, B.; Perveen, K.; Olsen, B.; Zahr, R. Emergence of carbapenem-resistant Acinetobacter baumannii in hospitals in Pakistan. J. Med. Microbiol. 2014, 63, 50–55. [CrossRef]

22. Shah, A.A.; Ali, Y.; Maqbool, A.; Abbasi, S.A. Phenotypic detection of extended-spectrum beta-lactamase in multidrug-resistant acinetobacter baumannii isolated in Fauji Foundation Hospital Rawalpindi. J. Pak. Med. Assoc. 2021, 71, 1144–1147. [CrossRef] [PubMed]

23. Khalid, F.; Saleem, S.; Ahmad, I. High prevalence of carbapenem-resistant Acinetobacter baumannii associated respiratory tract infections in Pakistanian hospitals. J. Pak. Med. Assoc. 2020, 70, 1630–1632. [CrossRef] [PubMed]

24. Strateva, T.; Sirakov, I.; Stoeva, T.; Stratev, A.; Dimov, S.; Savov, E.; Mitov, I. Carbapenem-resistant Acinetobacter baumannii: Current status of the problem in four Bulgarian university hospitals (2014–2016). J. Glob. Antimicrob. Resist. 2019, 16, 266–273. [CrossRef] [PubMed]

25. Liu, B.; Liu, L. Molecular Epidemiology and Mechanisms of Carbapenem-Resistant Acinetobacter baumannii isolates from ICU and Respiratory Department Patients of a Chinese University Hospital. Infect. Drug Resist. 2021, 14, 743–755. [CrossRef]

26. Waseem, H.; Ali, J.; Sarwar, F.; Khan, A.; Rehman, H.S.U.; Choudri, M.; Arif, N.; Subhan, M.; Saleem, A.R.; Jamal, A.; et al. Assessment of knowledge and attitude trends towards antimicrobial resistance (AMR) among the community members, pharmacists/pharmacy owners and physicians in district Sialkot, Pakistan. Antimicrob. Resist. Infect. Control 2019, 8, 67. [CrossRef]

27. Saleem, Z.; Godman, B.; Azzhar, F.; Kalungia, A.C.; Fadare, J.; Opana, S.; Markovic-Pekovic, V.; Hoxha, I.; Saeed, A.; Al-Gethamy, M.; et al. Progress on the national action plan of Pakistan on antimicrobial resistance (AMR): A narrative review and the implications. Expert Rev. Anti Infect. Ther. 2020, 20, 71–93. [CrossRef]

28. Vázquez-López, R.; Solano-Gálvez, S.G.; Juárez Vignon-Whaley, J.J.; Abello Vaamonde, J.A.; Paldró Alonzo, L.A.; Rivera Reséndiz, A.; Muleíro Álvarez, M.; Vega López, E.N.; Franyuti-Kelly, G.; Álvarez-Hernández, D.A.; et al. Acinetobacter baumannii Resistance: A Real Challenge for Clinicians. Antibiotics 2020, 9, 205. [CrossRef] [PubMed]

29. Ishitiaq, S.; Saleem, S.; Waheed, A.; Alvi, A.A. Molecular detection of blaOXA-23 gene and blaOXA-51 gene in carbapenem resistant strains of Acinetobacter baumannii in patients with ventilator associated pneumonia at tertiary care hospitals. J. Pak. Med. Assoc. 2021, 71, 2576–2581. [CrossRef]

30. Guo, J.; Li, C. Molecular epidemiology and decreased susceptibility to disinfectants in carbapenem-resistant Acinetobacter baumannii isolated from intensive care unit patients in central China. J. Infect. Public Health 2019, 12, 890–896. [CrossRef]

31. Ning, N.-Z.; Liu, X.; Bao, C.-M.; Chen, S.-M.; Cui, E.-B.; Zhang, J.-L.; Huang, J.; Chen, F.-H.; Li, T.; Qu, F.; et al. Molecular epidemiology of blaOXA-23-producing carbapenem-resistant Acinetobacter baumannii in a single institution over a 65-month period in north China. BMC Infect. Dis. 2017, 17, 14. [CrossRef]

32. Chang, Y.; Luan, G.; Xu, Y.; Wang, Y.; Shen, M.; Zhang, C.; Zheng, W.; Huang, J.; Yang, J.; Jia, X.; et al. Characterization of carbapenem-resistant Acinetobacter baumannii isolates in a Chinese teaching hospital. Front. Microbiol. 2015, 6, 910. [CrossRef]

33. Di Gennaro, F.; Marotta, C.; Amicone, M.; Bavaro, D.F.; Bernardo, F.; Frisicale, E.M.; Kurotschka, P.K.; Mazzari, A.; Veronese, N.; Murri, R.; et al. Italian young doctors’ knowledge, attitudes and practices on antibiotic use and resistance: A national cross-sectional survey. J. Glob. Antimicrob. Resist. 2020, 23, 167–173. [CrossRef]

34. Association, W.M. WMA Declaration of Helsinki—Ethical Principles for Medical Research Involving Human Subjects. Available online: https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/ (accessed on 8 May 2022).

35. CLSI. Performance Standards for Antimicrobial Susceptibility Testing, 2nd ed.; Clinical and Laboratory Standard Institute (CLSI): Wayne, PA, USA, 2002; Volume CLSI Supplement M100.

36. CLSI. Performance Standards for Antimicrobial Susceptibility Testing, 28th ed.; Clinical and Laboratory Standard Institute (CLSI): Wayne, PA, USA, 2018; Volume CLSI Supplement M100.
37. Shamsizadeh, Z.; Nikaeen, M.; Esfahani, B.N.; Mirhoseini, S.H.; Hatamzadeh, M.; Hassanzadeh, A. Detection of antibiotic resistant Acinetobacter baumannii in various hospital environments: Potential sources for transmission of Acinetobacter infections. *Environ. Health Prev. Med.* **2017**, *22*, 44. [CrossRef]

38. Ejaz, H.; Alzahrani, B.; Hamad, M.F.S.; Abosalif, K.O.A.; Junaid, K.; Abdalla, A.E.; Elamir, M.Y.M.; Aljaber, N.J.; Hamam, S.S.M.; Younas, S. Molecular Analysis of the Antibiotic Resistant NDM-1 Gene in Clinical Isolates of Enterobacteriaceae. *Clin. Lab.* **2020**, *66*, 409–417. [CrossRef]