DETECTION OF blaCTX-M AND blaSHV GENES OF EXTENDED SPECTRUM BETA LACTAMASE PRODUCING Klebsiella pneumoniae

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Abstract: Antimicrobial resistance poses a significant challenge in many hospitals, increasing infection morbidity and mortality. It is a global issue with far-reaching implications for human and animal health, the environment, agriculture, and the economy. The ineffective use of antibiotics in treating infectious diseases is a significant driver of antimicrobial resistance, which can be either innate or acquired. Objective: This study aimed to detect the presence of blaCTX-M and blaSHV genes in extended-spectrum beta-lactamase (ESBL) producing Klebsiella pneumoniae and to evaluate the antibacterial activity of zinc oxide (ZnO) nanoparticles synthesised using Calotrops procera extract. Methods: A cross-sectional study was conducted at Lahore College for Women University, Lahore, from January to December 2023. A total of 20 clinical isolates of Klebsiella pneumoniae were collected and identified using standard microbiological techniques. The presence of ESBL-producing strains was detected using the double disc synergy test. Polymerase chain reaction (PCR) was used to detect blaCTX-M and blaSHV genes. ZnO nanoparticles were synthesised using Calotrops procera extract and tested for antibacterial activity against ESBL-producing strains at different concentrations (20 mg/ml, 25 mg/ml, 30 mg/ml, and 35 mg/ml). The antibacterial activity was evaluated using the disc diffusion method, and data were analysed using SPSS version 23.0. Results: Of 20 clinical isolates, 15 (75%) were ESBL positive, and 5 (25%) were ESBL negative. PCR results showed that 80% of the ESBL-producing strains contained the blaCTX-M gene, and 83% included the blaSHV gene. The antibacterial activity of ZnO nanoparticles was concentration-dependent, with the highest inhibitory zones observed at a concentration of 35 mg/ml. The synergistic effect of ZnO nanoparticles with cefotaxime and ceftriaxone significantly increased the antibacterial activity against ESBL-producing strains. Conclusion: The study highlights the significant presence of blaCTX-M and blaSHV genes in ESBL-producing Klebsiella pneumoniae. The green synthesis of ZnO nanoparticles using Calotrops procera extract demonstrated enhanced antibacterial activity, especially when combined with beta-lactam antibiotics. These findings suggest that ZnO nanoparticles could be an alternative strategy to combat antimicrobial resistance.

Keywords: Antimicrobial Resistance, Beta-Lactamase, ESBL, Klebsiella Pneumoniae, Blactx-M, Blashv, Zinc Oxide Nanoparticles, Calotrops Procera, PCR, Synergistic Effect

Introduction

Antimicrobial resistance poses a significant challenge in many hospitals, increasing infection morbidity and mortality (1). It is a global issue with far-reaching implications for human and animal health, the environment, agriculture, and the economy (2, 3). The ineffective use of antibiotics in treating infectious diseases is a significant driver of antimicrobial resistance, which can be either innate or acquired (4, 5).

The spread of antibiotic resistance among clinical strains of various bacteria, including Acinetobacter, Klebsiella, Pseudomonas, and Escherichia coli, has become a significant concern (6). This resistance is often transmitted by Motile Genetic Elements (MGE) (7). The overuse of antibiotics has led to the emergence of multidrug-resistant bacterial strains, posing a severe threat to public health (8).

Furthermore, the discovery and development of antibiotics have been essential in combating microbial infections (9). However, the widespread use of broad-spectrum antibiotics has resulted in the emergence of multidrug-resistant isolates, complicating the clinical landscape. Antibiotics are classified based on their target, molecular structure, and mode of action, with different classes of antibiotics targeting cell membrane assembly, supermolecule synthesis, and nucleic acid synthesis (10, 11).

In addition to antibiotic resistance, beta-lactamases, enzymes produced by bacteria, present another challenge. These enzymes compete with beta-lactam antibiotics, such as penicillins and cephalosporins, rendering them ineffective by disrupting their molecular structure.

Addressing antimicrobial resistance and finding alternative strategies for combating bacterial infections are critical priorities for the global healthcare community.

Methodology

The study was conducted at the Department of Zoology, Lahore College for Women University, Lahore, to identify and characterise bacterial strains that produce beta-lactamases and assess their susceptibility to zinc oxide (ZnO) nanoparticles. Materials utilised in the study included various microbiological media such as Nutrient...
Agar, MacConkey Agar, Muller Hinton Agar (Oxoid), and Luria Bertani (LB) Broth (Sigma-Aldrich), along with antibiotic sensitivity discs for Imipenem (10 μg), Ciprofloxacin, Ceftriaxone, and Ampicillin (30 μg). Equipment used ranged from standard laboratory apparatus to specific molecular biology tools supplied by vendors, including Sartorius, Germany (balances); Capp, UK (micropipettes); and Memmert, USA (incubators).

For the synthesis of ZnO nanoparticles, zinc acetate (Sigma-Aldrich) was used, employing leaves of Calotropis procera as the biological precursor. Bacterial strains were collected from General Hospital, Lahore, from patient samples showing beta-lactamase activity. Fifteen strains, predominantly Klebsiella pneumoniae, were selected for further analysis.

All glassware and instruments were sterilised by autoclaving at 121°C and 15 psi for 20 to 30 minutes. Media were prepared according to the manufacturers’ specifications and autoclaved under the same conditions. The bacterial strains underwent morphological and biochemical identification based on protocols from Bergey’s Manual of Determinative Bacteriology. Molecular identifications and resistance mechanism investigations were conducted using PCR assays targeting blaCTX-M and blaSHV genes, optimised specifically for this study.

The strains’ antibacterial susceptibility to standard antibiotics and synthesised ZnO nanoparticles was assessed using the Kirby-Bauer disc diffusion method and healthy diffusion techniques, adhering to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The green synthesis of ZnO nanoparticles was carried out using a method described by Vidya et al. (2013) with Calotropis procera leaf extract, and the nanoparticles were characterised using spectroscopic techniques.

Data from the study were analysed using Microsoft Excel 2010 and SPSS version 16.0, with the statistical significance of differences in inhibition zones determined by one-way ANOVA and post-hoc tests. The Institutional Review Board of Lahore College for Women University granted ethical approval for the study, ensuring compliance with ethical standards for research involving bacterial isolates from human sources.

Results

Klebsiella pneumonia colonies appeared to be large and mucoid. Following gram staining, the colonies were coloured pink, showing that they were gram-negative rods. In the catalase test, K. pneumoniae formed bubbles, indicating reduced hydrogen peroxide. There was no response from K. pneumoniae in an oxidase test. The indole test came up negative.

The double disc synergy test (Jarlier et al., 1988) was used to detect ESBL-generating gram-negative bacterial strains. Figure 4.3 shows that after phenotypic identification of ESBLs, 15 (75%) out of 20 clinical isolates were ESBL positive, while 5 (25%) were ESBL negative.

Gram-negative bacterial strains were tested to see how ZnO nanoparticles in DMSO at various doses (20 mg/ml, 25 mg/ml, 30 mg/ml, and 40 mg/ml) affected them. The maximum inhibitory zones were attained at a 40 mg/ml nanoparticle concentration. Figures and tables show how nanoparticles have antibacterial properties. This was also seen on the graph, where the maximum bar was produced at a concentration of 40 mg/ml nanoparticles and the lowest at 20 mg/ml.

Table 4.1 shows the zones of inhibition formed when ZnO nanoparticle concentrations of 20mg/ml, 25mg/ml, 30mg/ml, and 35 mg/ml were used. The results showed that at 35 mg/ml concentration of ZnO NPs, K. pneumoniae (1245) had the highest zone of inhibition of 13mm, and K. pneumoniae (336) had the lowest zone of inhibition of 7mm.

![Fig. 4: Detection of ESBLs Producing Strains](image)

![Fig. 5: Antimicrobial activity of ZnO Nanoparticles against bacterial strains](image)

Where: A: 20 mg/ml of ZnO, B: 25 mg/ml of ZnO, C: 30 mg/ml of ZnO, D: 35 mg/ml of ZnO, E: Negative Control (water), S: Sensitive antibiotic (Imipenem) and R: Resistant antibiotic (Ciprofloxacin)

### Table 1: Antimicrobial activity of ZnO nanoparticles against bacterial strains by disc diffusion method

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Zone of inhibition in mm</th>
</tr>
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<tbody>
<tr>
<td>Concentration of ZnO NPs (mg/ml)</td>
<td>Antibiotics</td>
</tr>
<tr>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>K. pneumoniae (336)</td>
<td></td>
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<tr>
<td>K. pneumoniae (08)</td>
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<td>K. pneumoniae (1295)</td>
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<td>K. pneumoniae (1253)</td>
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<td>K. pneumoniae (1285)</td>
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<td>K. pneumoniae (358)</td>
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<td>K. pneumoniae (1254)</td>
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<tr>
<td>K. pneumoniae (310)</td>
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<td>K. pneumoniae (1133)</td>
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<td>K. pneumoniae (1295)</td>
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</table>

K. pneumoniae (310) 8±0.4 10±0.4 12±0.4 13±0.4 16±0.2 R
K. pneumoniae (1253) 9±0.3 11±0.5 12±0.3 12±0.2 25±0.1 R
K. pneumoniae (336) 7±0.3 10±0.4 11±0.5 14±0.3 16±0.5 R
K. pneumoniae (1245) 7±0.3 8±0.5 10±0.3 11±0.5 30±0.3 R
K. pneumoniae (358) 8±0.4 10±0.4 12±0.4 13±0.4 20±0.2 R
K. pneumoniae (1285) 13±0.5 14±0.3 15±0.5 16±0.2 25±0.5 R
K. pneumoniae (1241) 8±0.4 9±0.4 10±0.3 11±0.3 26±0.4 R
K. pneumoniae (08) 9±0.3 11±0.5 13±0.4 15±0.5 20±0.3 R
The increased antibacterial properties of the nanoparticle with drugs including cefotaxime, ceftriaxone, and ciprofloxacin against particular bacterial strains were also assessed. The outcomes demonstrated that the presence of ZnO NPs improved the antibacterial activity of cefotaxime and ceftriaxone. The outcome demonstrated that antibiotics and NPs work together to produce greater antibacterial effects. The table data were presented according to zone of inhibition.

![Image of bacterial strain plates with labeled zones of inhibition](image1)

**Fig. 7: Synergistic effect of Nanoparticles against bacterial strains**

Where: A: Control, B: CTX, C: CRO, D: Nanoparticles, E: CTX+ Nanoparticles, F: CRO+Nanoparticles and R: Resistant antibiotic (Ciproflaxcin)

![Graphical presentation of Synergistic effect of nanoparticles with antibiotics (CRO)](image2)

**Fig 8: Graphical presentation of Synergistic effect of nanoparticles with antibiotics (CRO)**

The alkaline lysis method was used to isolate plasmid DNA. An ultraviolet (UV) light source, such as a transilluminator 1, is used to excite the fluorescent molecules in order to visualize the DNA segments. Ethidium Bromide (EtBr) is the most commonly used to fluorescent DNA. Intercalation occurs when EtBr molecules squeeze between neighboring base pairs in a DNA double helix. Any EtBr intercalated into the DNA fluoresces and emits a bright orange light when exposed to UV light. Figure 4.14 shows the bacterial bands on an agarose gel illuminated by a UV Tran illuminator. DNA was isolated from 20 clinical isolates. DNA was visible in 10 clinical isolates only. To determine the prevalence of blaCTX-M gene, PCR was performed on bacterial strains that were ESBLs positive. A total of 8 (80%) clinical strains contained the gene, while 2 (20%) clinical isolates did not contain the blaCTX-M. The size of

the PCR product was estimated using DNA markers and a known DNA ladder. For blaCTX-M, the product size observed on gel was approximately 600bp.

![Graphical presentation of Synergistic effect of nanoparticles with antibiotics (CTX)](image)

![Visualization of plasmid DNA of clinical isolates](image)

![PCR Amplification for BlaCTXM gene](image)

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To determine the prevalence of the SHV gene, PCR was performed on bacterial strains that were ESBLs positive. A total of 6 (83%) clinical strains contained the CTX-M gene, while 2 (20%) clinical isolates did not contain the SHV gene. The size of the PCR product was estimated using DNA markers and a known DNA ladder. For, SHV gene the product size observed on gel was approximately 716bp.

**Discussion**

The current study was designed to isolate and characterize the blaSHV and blaCTX-M gene and green synthesis s of ZnO using Calotropis procera extract. These nanoparticles were tested for antibacterial activity against gram negative bacterial strains such as *K. pneumoniae* which were sensitive to the beta-lactam antibiotic Imipenem but resistant to Ciprofloxacn. The double disc test was used to identify ESBLs-producing strains. Bacterial susceptibility to nanoparticles was tested using a disc diffusion assay with four different nanoparticle concentrations (20mg/ml, 25 mg/ml, 30 mg/ml, and 35 mg/ml). When observed individually, NPs showed antibacterial effects as indicated by the zone of inhibition, but the beta-lactam antibiotics cefotaxime and ceftriaxone comparatively enhanced this effect. The highest zones of inhibition were obtained at the highest concentration of nanoparticles, 35 mg/ml. The difference between the means seemed statistically significant at p ≤ 0.05.

Antibiotic resistance was now recognised as a widespread issue that affects both hospital-acquired diseases and non-hospital strains that could be treated with antibiotics. Numerous MDR-based infections not only reduce the effectiveness of current treatments but also result in thousands of fatalities. To address this issue, rapid modifications were required (12). Carbenem resistance Enterobacteriaceae (CRE) was increasingly isolated from community-acquired and nosocomial infections (13, 14). CRE could spread from person to person, and the genes encode carbapenems could spread horizontally between strains (15). As a result of its ease of spread, it has become a significant public health concern. Previous research has shown that plasmids carrying the carbapenemase gene frequently carry resistance genes to other antibiotics (16).

Beta-lactam antibiotics, commonly used to treat bacterial infections, were becoming less effective against Enterobacteriaceae. The production of ESBLs by these bacteria is the primary cause of this decrease in efficacy (17). Various beta-lactamases have emerged due to the widespread use of beta-lactam antibiotics in clinical practice over the last several decades. Production was the most common resistance mechanism to beta-lactam antibiotics in gram-harmful bacteria. The production of ESBLs facilitated beta-lactamase enzyme resistance to third-generation cephalosporins (18), BlaTEM type ESBLs had recently increased rapidly in Enterobacteriaceae, *K. pneumoniae*, and *E. coli*. In the current study, 83% of *K. pneumonia* producing ESBLs tested positive for the blaSHV and 80% for blaCTX-M genes present. (Figure 16, 17). These values were similar to those found in another study. Another study from Pakistan found that only 53% of bacterial isolates had the blaSHV and blaCTX-M genes, whereas our study found a higher incidence. Our findings demonstrated that the rate of ESBL-producing *K. pneumoniae* harbouring the blaSHV and blaCTX-M genes increased rapidly. In 2005, the values for the blaSHV and blaCTX-M genes were 4% and 43%, respectively. This rising trend was also mentioned in several other comparative studies (19).

According to the findings of this study, the most common ESBL gene found in *K. pneumoniae* isolates was blaCTX-M. Antimicrobial susceptibility testing of *K. pneumoniae* isolates producing 80% ESBLs. Most continents were concerned about the global spread of blaCTX-M-producing *K. pneumoniae*. According to Eskandari-Nasab et al. (2018), the prevalence of blaCTX-M was 10.0, 30.0, 35.3, 56.7, 64.4, 96.9, and 100.0% in Bahrain, Turkey, Saudi Arabia, Iran, United Arab Emirates, Pakistan and Kuwait, respectively (20). While international studies found varying percentages of this gene present in ESBL isolates from North Africa, America, Russia, Latin America, Brazil, and Europe, the percentages were 7.4, 26.4, 34.9, 61.1, 62.1, and 84.5%, respectively, according to Raouf et al., (2022). Even though blaTEM and blaSHV variants were the most common ESBLs, they appear to have become less common over the last decade than blaCTX-M. The findings of this study were consistent with previous studies that found the blaCTX-M gene to be the most common ESBL type in *K. pneumoniae* isolates. In contrast to our findings, Ferreira et al. (2019) from Brazil and Kerluku et al. (2023) from Portugal found a higher prevalence of blaSHV than blaCTX-M in *K. pneumoniae* (21, 22). Many factors could

contribute to these differences, including sample origin, sample size, studied population, and detection methods. Finally, our findings revealed the presence of ESBL genes in 80% of K. pneumoniae isolates. Previous research from Brazi 110, China 16, 17, and Portugal18 found multiple ESBL genes in clinical isolates of K. pneumoniae. According to our findings, green-synthesized ZnO outperformed chemically synthesised ZnO regarding antimicrobial activity. Chemical composition, concentration, size, shape, and photo-activation were some of the factors that influence metallic nanoparticle antimicrobial properties. Because the composition and size of chemical and green synthesised NP differ, they exhibit different antimicrobial activity against the same pathogens. Antibiotics were tested for antimicrobial activity against the bacterial strains used in the study. Imipenem was the only antibiotic that was effective against all strains. K. pneumoniae was more sensitive to Imipenem with a maximum zone of inhibition of 30mm. A study by Bradley et al., 2016 found that meropenem was slightly more active against gram-positive than gram-negative pathogens. Unlike Imipenem, all bacterial strains were resistant to the antibiotic ciprofloxacin, with no zone of inhibition observed. Aliakbar Nasiri et al. (2016) conducted a study in which 20 strains of P. aeruginosa were isolated, and their susceptibility to different antibiotics was tested, one of which is Ciprofloxacin, and all strains were resistant to ciprofloxacin. Zhang et al. (2009) recommended the potential mechanisms involving the interaction of nanomaterials with biological molecules. The author documented that the negative and positive charges of microorganisms and metal oxides create an electromagnetic attraction between the microbe and the treated surface. When the contact is made, the microbe is oxidised and dead instantly.

According to Glover et al. (2006), among many other NPs, ZnO NPs were being studied for their photocatalytic antimicrobial activity. In the current study, green synthesised ZnO had the highest zone of inhibition, 12mm, with disc diffusion assay among all strains tested at the highest concentration, 35 mg/ml. Haghi et al. (2011) used the double disc diffusion test to examine the antibacterial effect of 0.01, 0.5, 1, and 1.5% ZnO NPs on K. pneumoniae. The maximum 5mm zone of inhibition was observed in 1.5% ZnO NPs. The current study observed a maximum inhibition zone of 12mm against K. pneumoniae 35mg/ml of ZnO NPs, Russell et al. (1994) discovered that a substantial hindrance of nanoparticles to the outer membrane inhibits active transport, dehydrogenase and periplasmic enzyme activity and eventually inhibits RNA. The synthesis of DNA and proteins caused cell lysis. The current study showed that ZnO nanoparticles had a synergistic effect with the antibiotics imipenem, ciprofloxacin, ceftriaxone, ceftriaxone, ceftriaxone, ceftriaxone, ceftriaxone, i.e., they formed a zone of inhibition against all the bacterial strains. Ciprofloxacin, ceftriaxone, and cefotaxime had no inhibitory activity when used alone, but when combined with ZnO, the zone of inhibition was increased against K. pneumoniae.

Alekish et al. (2018) investigated the effect of ZnO nanoparticles with different antibiotics against MDR S. aureus (23). Among the antibiotics, cefotaxime formed a zone of inhibition of 20mm when used alone but 24mm when combined with ZnO nanoparticles. Their findings may be related to the current findings, which show a synergistic effect of cefotaxime and ZnO nanoparticles. When ZnO nanoparticles were combined with the beta-lactam antibiotics cefotaxime and ceftriaxone, the antimicrobial activity of these antibiotics against all bacterial isolates was increased. The zone of inhibition was 18mm (ZnO-CTX) and 15mm (ZnO+CRO) against K. pneumoniae (358). Other nanoparticles have been studied similarly. Nazari et al. (2012) conducted a similar study in which gold NPs combined with cefotaxime antibiotics increased the antibacterial effect of antibiotics at test concentration (40g/disc). In this study, 20 clinical isolates were initially screened for carbapenemase production. In our current study, ESBLs in clinical isolates yielded 75% favourable and 25% negative results. Various studies conducted by multiple researchers showed different results such as Jacobson showed lower ESBL production in P. aeruginosa, 7.7%, 4.2%, 3.7%, and 9.2%, respectively (24, 25). The rapid spread of blaCTX-M and blaSHV-producing Enterobacteriaceae, particularly K. pneumoniae, was a worldwide public health concern. An earlier study from another Nepalese tertiary care hospital found that respiratory tract specimens had a lower incidence of producing gram-negative bacteria (1.3%). (26). Another recent news report from Brazil was reported in 2008. According to Alkhudhaire et al., 38.9% of gram-negative bacterial isolates tested positive for blaCTX-M and 29.8% for blashv positive (27). This study produced similar results because all blaCTX-M and blash-positive strains were gram-negative bacteria. Antibacterial activity increases with the increase in concentration of nanoparticles. Therefore, we use ZnO NPs to hinder the growth of bacteria. This study will be helpful for the formulation of novel antibacterial agents that were required to develop a generation of therapies to combat antimicrobial resistance. The antibacterial properties of these nanoparticles could be explored for use in various medical and industrial applications, such as the renewal of the antibiotic development strategy to overcome antimicrobial resistance. Antimicrobial awareness campaigns at the country level should be established, suggesting that DNA sequence analysis should be done to check the nucleotide sequence changes in blaCTX-M and blashv due to ZnO nanoparticles.

**Conclusion**

Bacteria are becoming increasingly resistant to antibiotics. This study emphasises the significance of the growing threat posed by ESBL-producing K. pneumoniae. This results in resistance to third-generation cephalosporins such as cefotaxime and ceftriaxone. Calotropis procera-derived ZnO nanoparticles are more durable, eco-friendly, cheap, and affordable. The antimicrobial activity of biosynthesised ZnO nanoparticles increased with increasing surface-to-volume ratio due to a decrease in particle size of nanoparticles. ZnO nanoparticles improved the efficacy of previously resistant antibiotics CTX and CRO against ESBL-positive bacterial strains. That is the conclusion. At various concentrations, ZnO nanoparticles can inhibit the growth of gram-negative bacteria such as K. pneumoniae.
Recommendations

Antibacterial activity increases with the increase in concentration of nanoparticles. Therefore, we use bio-synthesized zinc oxide NPs to hinder the growth of bacteria. This study will be helpful for the formulation of novel antibacterial agents that are required to develop a new generation of therapies to combat anti-microbial resistance. The antibacterial properties of these nanoparticles can be explored in the future for use in various medical and industrial applications—renewal of the antibiotic development strategy to overcome antimicrobial resistance. Antimicrobial awareness campaigns at the country level should be established. It is suggested that DNA sequence analysis should be done to check the nucleotide sequence changes in blaCTX-M and blaSHV due to ZnO nanoparticles.

Declarations

Data Availability statement
All data generated or analyzed during the study are included in the manuscript.

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Approved by the department concerned. (IRB/no-23339/LCWL dated 5-2-22)

Consent for publication
Approved

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Study Design, Review of Literature.

SARA MAHMOOD
Conception of Study, Final approval of manuscript.

MAH PARA
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IQRA TABASSUM
Manuscript drafting.

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Manuscript revisions, critical input.

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