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Original Article

Microbiological Profile and Antibiotic Resistance Patterns of Bacteria Isolated from Extruded Lumbar Discs in Patients Undergoing Lumbar Discectomy

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Background:.Lumbar intervertebral disc herniation and degenerative disc disease have traditionally been considered consequences of mechanical stress and degenerative changes. However, emerging evidence suggests that low-grade bacterial infection may contribute to disc pathology and inflammation. This study aimed to evaluate the microbiological profile and antimicrobial resistance patterns of bacteria isolated from extruded lumbar disc specimens obtained from patients undergoing lumbar discectomy

Materials and Methods: In this cross-sectional study, 115 patients undergoing lumbar discectomy were evaluated. Demographic data, clinical characteristics, and MRI findings were recorded. Disc samples were collected intraoperatively under sterile conditions and immediately transported for microbiological analysis. Specimens were homogenized and cultured on blood agar, MacConkey agar, and thioglycollate broth under aerobic and anaerobic conditions at 37°C. Isolated colonies were identified using standard microbiological techniques. PCR analysis targeting 16S rRNA gene was performed on selected bacterial isolates. Antimicrobial susceptibility testing was conducted according to CLSI guidelines, and multidrug resistance patterns were assessed

Results: The mean age of patients was 44.7 ± 5.76 years, and 60% were male. The mean BMI was 30.7 ± 5.2 kg/m². Spondylolisthesis (38.3%) was the most frequent MRI finding. Microbiological analysis revealed no bacterial growth in 62.6% of samples. Among positive cultures, anaerobic bacteria (9.5%) were the most common isolates, followed by coagulase-negative staphylococci (6.1%) and diphtheroids (6.1%). *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, Bacillus, and Coryneform bacteria were detected at lower frequencies. Antimicrobial susceptibility testing demonstrated multidrug resistance patterns, particularly among *E. coli* and *P. aeruginosa*, with concerning carbapenem resistance observed in Pseudomonas isolates. Methicillin-resistant *S. aureus* (MRSA) accounted for approximately 40%.

Conclusion: A proportion of extruded lumbar disc samples demonstrated bacterial presence, predominantly low-virulence and anaerobic organisms. The observed antimicrobial resistance patterns highlight potential clinical concerns. These findings support the hypothesis that bacterial involvement may contribute to disc pathology in a subset of patients; however, further large-scale studies using advanced molecular techniques are required to clarify causality

Keywords Lumbar disc herniation; intervertebral disc; Bacterial infection; PCR; Microbiological culture

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Introduction

Lumbar disc herniation (LDH) is among the most common causes of chronic low back pain and radiculopathy worldwide and represents a major contributor to disability and healthcare burden (1). Intervertebral disc degeneration (IVDD), which underlies many cases of LDH, has traditionally been attributed to age-related degeneration, mechanical stress, genetic predisposition, obesity, smoking, and repetitive spinal loading (2). Despite advances in spinal imaging and surgical techniques, the exact pathophysiological mechanisms contributing to disc degeneration and herniation remain incompletely understood (3).

In recent years, increasing attention has been directed toward the potential role of low-grade bacterial infection in the pathogenesis of degenerative disc disease (5). Several studies have demonstrated the presence of bacterial DNA and viable microorganisms within excised intervertebral disc tissue obtained during spinal surgery. Among these microorganisms, *Cutibacterium acnes* (formerly *Propionibacterium acnes*) has been the most frequently reported species, although coagulase-negative staphylococci, *Staphylococcus aureus*, Corynebacterium species, and Gram-negative bacilli have also been identified (6). The proposed mechanism underlying bacterial involvement in disc pathology is biologically plausible. Disc herniation and annular disruption may facilitate bacterial translocation into the relatively immune-privileged intervertebral disc environment through hematogenous spread or microvascular invasion (7). Once established, low-virulence bacteria may induce chronic inflammation through activation of pro-inflammatory cytokines, matrix metalloproteinases, and immune-mediated degenerative pathways, thereby accelerating disc degeneration and contributing to pain generation (8). Experimental studies have further suggested that bacterial colonization may promote Modic changes and inflammatory responses within adjacent vertebral endplates (9). Nevertheless, the role of bacteria in IVDD and LDH remains controversial (10).

While multiple systematic reviews and meta-analyses have reported bacterial positivity rates ranging from approximately 25% to 36% in surgically excised disc specimens, other investigations have argued that many positive cultures may represent perioperative contamination rather than true intradiscal infection (11). Differences in sampling techniques, culture conditions, molecular diagnostic methods, and contamination control strategies have contributed substantially to inconsistencies across studies (12).

In addition to the controversy regarding bacterial presence, limited data are available concerning the antimicrobial resistance profiles of bacteria isolated from intervertebral disc specimens. This issue is clinically important because the emergence of multidrug-resistant (MDR) organisms, including methicillin-resistant *S. aureus* (MRSA) and carbapenem-resistant Gram-negative bacteria, poses significant therapeutic and epidemiological challenges worldwide. Understanding the microbiological spectrum and resistance patterns of bacteria associated with lumbar disc pathology may provide valuable insights into disease mechanisms and future therapeutic strategies (13, 14, 15).

Therefore, the present study aimed to evaluate the microbiological profile and antibiotic resistance patterns of bacteria isolated from extruded lumbar disc specimens obtained from patients undergoing lumbar discectomy. In addition, molecular detection using polymerase chain reaction (PCR) targeting the bacterial 16S rRNA gene was employed to improve diagnostic accuracy and further investigate bacterial involvement in lumbar disc disease.

Materials and methods

Study Design and Setting

This cross-sectional study was conducted over a one-year period (April 2025 to April 2026) to evaluate the microbiological profile and antimicrobial resistance patterns of bacteria isolated from extruded lumbar intervertebral disc specimens obtained from patients undergoing lumbar discectomy at Babol University of Medical Sciences, Rouhani Hospital, Babol, Iran. The study was performed in collaboration between the Department of Neurosurgery and the Microbiology Laboratory of Rouhani Hospital.

All patient information was treated confidentially throughout the study. Data were analyzed and reported anonymously, and no personally identifiable information was disclosed in any publication or report derived from this research. Written informed consent was obtained from all participants prior to enrollment. The study protocol was approved by the Ethics Committee of Babol University of Medical Sciences under the ethics approval code

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Study Population

The study population consisted of patients diagnosed with LDH who underwent lumbar discectomy during the study period. The diagnosis of LDH was established by a neurosurgeon based on clinical manifestations and magnetic resonance imaging (MRI) findings.

Eligibility Criteria

Patients were eligible for enrollment if they met all of the following criteria: presence of extruded LDH requiring lumbar discectomy, admission to Rouhani Hospital, Babol, radiological confirmation of lumbar disc herniation by MRI, confirmation of diagnosis by a neurosurgeon availability of complete medical records and Provision of written informed consent. So, cases were excluded in the presence of any of the following conditions: undergoing neurosurgical procedures other than lumbar discectomy, antibiotic consumption within two weeks prior to surgery, presence of fever, acute or chronic infection, or inflammatory disease during the preceding two weeks and contaminated or inadequate specimens for microbiological analysis.

Sampling Method and Data Collection

Participants were recruited using a consecutive convenience sampling method. All eligible patients undergoing lumbar discectomy during the study period were consecutively enrolled until the required sample size was achieved. Demographic and clinical data, including age, sex, body mass index (BMI), comorbidities, previous surgical history, and MRI findings, were extracted from patients' medical records and recorded using a standardized study checklist.

Specimen Collection

All specimens were collected intraoperative under strict aseptic conditions to minimize the risk of contamination from normal skin flora. Prior to surgery, the operative field was prepared according to a standardized sterilization protocol using two sequential applications of 70% isopropyl alcohol followed by three applications of povidone–iodine solution. Following complete surgical field preparation, tissue samples including extruded disc material, adjacent muscle tissue, and skin scraping specimens were obtained by the neurosurgeon using sterile instruments. Each specimen was immediately transferred into separate sterile Falcon tubes and transported promptly to the microbiology laboratory for further analysis. To avoid cross-contamination, separate sterile instruments were used for each specimen, and unnecessary environmental exposure was strictly minimized throughout the sampling process.

Microbiological Analysis

Upon arrival at the microbiology laboratory, specimens were processed immediately under sterile conditions within a laminar airflow cabinet. Tissue samples were mechanically homogenized using sterile surgical blades and homogenizers to enhance bacterial release from tissue matrices and facilitate uniform culture inoculation. Homogenized samples were inoculated onto 5% sheep blood agar, MacConkey agar, and chocolate agar plates. Blood agar was used for the isolation of a broad spectrum of Gram-positive and Gram-negative bacteria and assessment of hemolytic activity. MacConkey agar served as a selective and differential medium for Gram-negative enteric bacilli based on lactose fermentation characteristics.

Chocolate agar was utilized for fastidious organisms requiring enriched nutritional conditions. Aerobic cultures were incubated at 37°C for 24–48 hours under aerobic conditions. In the absence of visible growth, incubation was extended up to 72 hours. Notably, turbidity observed in thioglycollate broth was considered only as a presumptive indicator of anaerobic growth. Due to technical limitations, including the lack of a complete anaerobic culture system and gas-pack facilities, definitive isolation and species-level identification of anaerobic bacteria could not be performed. Cultivable Bacterial isolates were identified based on colony morphology, Gram staining, and standard biochemical tests, including catalase, coagulase, oxidase, TSI, SIM, citrate, urease, and hemolysis pattern analysis. Gram-positive and Gram-negative bacteria were differentiated using conventional microbiological algorithms. For molecular confirmation, representative colonies from culture-positive specimens underwent PCR targeting the bacterial 16S rRNA gene using universal primers. PCR amplification of the bacterial 16S rRNA gene was performed on selected isolates using the universal bacterial primers 27F (5′-AGAGTTTGAT CMTGGCTCAG-3′) and 1492R (5′-TACGGYTACC TTGTTACGACTT-3′). Each PCR reaction was carried out in a final volume of 25 µL containing PCR master mix, primers, template DNA, and

nuclease-free water. Amplification was performed under the following conditions: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s, with a final extension at 72°C for 7 min. PCR products were analyzed by agarose gel electrophoresis. Positive and negative controls were included in each run to validate amplification and monitor contamination. Selected PCR products were subsequently subjected to sequencing, and the obtained sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database to confirm bacterial species identification based on sequence similarity.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed for pathogenic clinically significant isolates using the Kirby–Bauer disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI; 2024). Antibiotics were selected based on organism type and routine clinical microbiology protocols. Multidrug resistance (MDR) was defined as resistance to at least one antimicrobial agent in three or more antibiotic classes. Methicillin resistance among *S.aureus* isolates was determined using cefoxitin disk (fox 30 µg) testing according to CLSI criteria.

Statistical Analysis

Data were analyzed using SPSS software version 26. Quantitative variables were expressed as mean ± standard deviation (SD), whereas categorical variables were presented as frequencies and percentages.

Results

Demographic and Clinical Characteristics

The sample size was determined based on the study by Withanage et al. (2019) (7) and using the formula $n = z^2P(1-P)/d^2$, where n denotes the sample size, P represents the estimated prevalence derived from a pilot study, z corresponds to the 95% confidence level, and d indicates the acceptable margin of error (0.05). Based on this calculation, a minimum sample size of 103 non-duplicate and non-consecutive clinical specimens was required. Ultimately, 115 patients undergoing lumbar discectomy were included in the present study.

The mean±SD age of participants was 44.7 ± 5.76 years, with the highest proportion of patients belonging to the 37–50-year age group (42.6%). Male patients constituted 60% of the study population. The mean body mass index (BMI) was 30.7 ± 5.2 kg/m², and the majority of patients were classified within the overweight category (BMI 25–30 kg/m²). MRI findings demonstrated that spondylolisthesis was the most common radiological abnormality, identified in 38.3% of patients, followed by disc protrusion. Previous lumbar surgery was reported in 15.6% of cases. Regarding comorbidities, cigarette smoking was the most prevalent associated factor (28.6%), whereas 38.2% of patients had no documented underlying disease **(Table 1)**. Some patients had more than one MRI finding.

Microbiological Findings

Extruded lumbar disc specimens obtained during surgery were processed immediately under strict aseptic laboratory conditions. Following tissue homogenization, samples were cultured on blood agar, MacConkey agar, and thioglycollate broth for aerobic and anaerobic microbiological assessment. Overall, no microbial growth was detected in 62.6% of specimens. Among culture-positive samples, anaerobic bacteria represented the most frequently identified microorganisms (9.5%), followed by coagulase-negative staphylococci (CoNS) and diphtheroid organisms, each accounting for 6.1% of isolates. Lower isolation rates were observed for *S. aureus* (4.3%), coryneform bacteria (5.2%), Bacillus spp. (2.6%), *E. coli* (1.7%), and *P. aeruginosa* (1.7%) **(Fig 1).**

The distribution of isolated microorganisms among extruded disc specimens was statistically significant according to the chi-square test ($P < 0.001$), indicating a non-random microbiological pattern within the studied samples. Notably, turbidity observed in thioglycollate broth cultures was considered indicative of anaerobic bacterial growth. Due to technical limitations, including lack of access to a complete anaerobic culture system and gas-pack facilities, definitive anaerobic species isolation and characterization could not be performed. PCR amplification targeting the bacterial 16S rRNA gene was performed on representative colonies obtained from culture-positive specimens. Molecular analysis was carried out using universal 16S rRNA primers to confirm bacterial identification. It should be noted that PCR analysis was not performed for anaerobic isolates due to the inability to recover pure anaerobic strains, nor for diphtheroid organisms, Bacillus spp., and coryneform

bacteria because these microorganisms were considered part of the normal skin or environmental flora and were therefore excluded from molecular confirmation. All bacterial colonies subjected to molecular analysis were successfully confirmed by 16S rRNA PCR and subsequent sequence analysis using the BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) database, demonstrating high sequence similarity with the corresponding bacterial species.

Table 1 Demographic and Clinical Characteristics of Patients Undergoing Lumbar Discectomy

Variable	Category	N (%)	Mean ± SD
Age (years)	< 25	8 (6.9)	44.7 ± 5.76
	26–36	31 (27.0)	
	37–50	49 (42.6)	
	> 50	27 (23.5)	
Sex	Male	69 (60.0)	--
	Female	46 (40.0)	
BMI (kg/m²)	< 25	24 (20.8)	30.7 ± 5.2
	25–30	47 (40.8)	
	> 30	44 (38.2)	
MRI Findings	Disc protrusion	29 (25.1)	--
	Disc extrusion	18 (15.6)	
	Spondylodiscitis	3 (2.6)	
	Spondylolisthesis	44 (38.3)	
	Degenerative disc disease	21 (18.3)	
Previous Surgery	Yes	18 (15.6)	--
	No	97 (84.3)	
Comorbidities	Diabetes mellitus	21 (18.2)	--
	Hypertension	17 (14.7)	
	Smoking	33 (28.6)	
	No comorbidity	44 (38.2)	

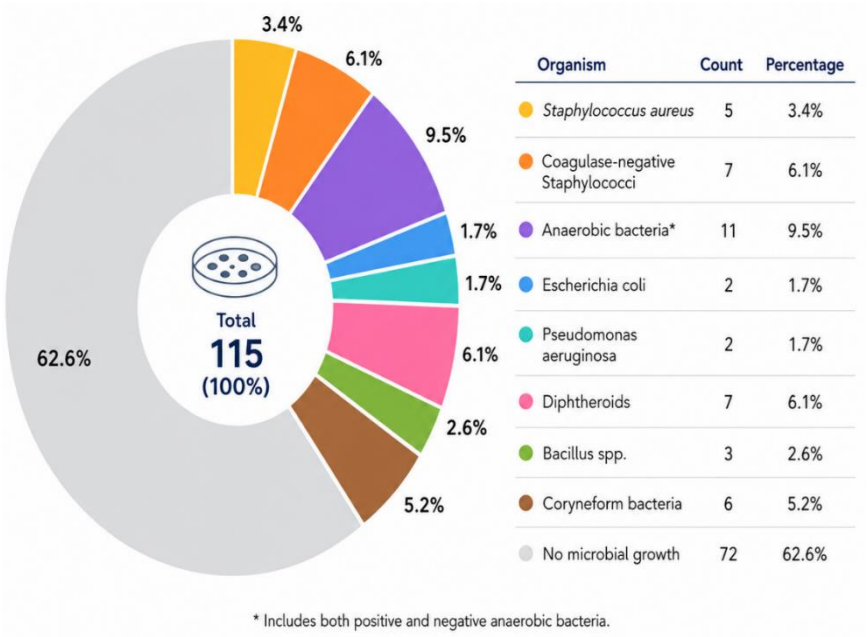

Fig. 1. Distribution of isolated organisms

Antimicrobial Resistance Patterns

Among *S. aureus* isolates, resistance to cefoxitin was detected in 40% of cases, suggesting the presence of MRSA. CoNS isolates also exhibited substantial resistance to β-lactam agents and cephalosporins, indicating a relatively high prevalence of MDR phenotypes within this group. In *E. coli* isolates, high resistance rates to ampicillin and ceftriaxone were observed, while reduced susceptibility to imipenem in a subset of isolates raised concerns regarding emerging carbapenem resistance. Similarly, 50% of *P. aeruginosa* isolates demonstrated resistance to imipenem, consistent with the emergence of carbapenem-resistant *P. aeruginosa* (CRPA). Detailed antimicrobial susceptibility patterns of the isolated clinical strains are presented in **(Table 2).**

Table 2. Antimicrobial Susceptibility Patterns of Clinical Isolates Recovered from Extruded Lumbar Disc Specimens

Antimicrobials agents	N (%) of resistance isolates											
	P. aeruginosa(N; 2			S. aureus(N; 5)			CoNS (N;7)			E. coli(N;2)		
	R	I	S	R	I	S	R	I	S	R	I	S
LEX	1 (50%)	0	1 (50%)	1 (50%)	0	1 (50%)	3 (42.8%)	1 (14.3%)	3 (42.8%)	2 (40%)	0	3 (60%)
GM	1 (50%)	0	1 (50%)	0	0	2 (100%)	4 (57.1%)	0	3 (42.8%)	2 (40%)	0	3 (60%)
SXT	1 (50%)	0	1 (50%	2 (100%)	0	0	4 (57.1%)	0	3 (42.8%)	3 (60%)	1 (20%)	1 (20%)
AMP	0	0	2 (100%)	1 (50%)	0	0	5 (57.1%)	0	2 (28.5%)	4 (80%)	0	1 (20%)
IPM	1 (50%)	0	1 (50%)	0	0	2 (100%)	-	-	-	-	-	-
FOX	-	-	-	-	-	-	4 (57.1%)	0	3 (42.8%)	2 (40%)	1 (20%)	2 (40%)
CRO	1 (50%)	0	0	0	0	2 (100%)	2 (28.5%)	1 (14.3%)	4 (57.1%)	1 (20%)	0	4 (80%)

S, susceptible; I, intermediate; R, resistant; CoNS, coagulase-negative staphylococci, Cephalexin, LEX; Gentamicin, GM; Trimethoprim/Sulfamethoxazole, SXT; Ampicillin, AMP, Imipenem, IPM; Cefoxitin, FOX; Ceftriaxone, CRO.

Discussion

The demographic characteristics observed in the present study are consistent with the established epidemiology of lumbar disc disease. The mean age of patients was approximately 45 years, with the highest prevalence observed between 37 and 50 years of age. This aligns with previous epidemiological studies and spine registry data reporting peak incidence of symptomatic lumbar disc degeneration and herniation during the fourth and fifth decades of life, which is attributed to progressive intervertebral disc dehydration, extracellular matrix breakdown, and cumulative biomechanical stress over time (16–18). Repetitive axial loading and age-related structural changes contribute to annular weakening and disc instability, thereby increasing susceptibility to disc herniation and degenerative spinal disorders (17,18).

Male predominance in the present cohort is also consistent with multiple international studies demonstrating higher rates of lumbar disc pathology in men (4). Occupational exposure to heavy physical workload, repetitive lifting, spinal microtrauma, and lifestyle factors such as smoking may partly explain this difference (19, 20). However, sex-related differences in lumbar disc degeneration remain multifactorial and may vary according to geographic, occupational, and population-specific characteristics (21). Another important finding in this study was the relatively high BMI among patients, with most individuals classified as

overweight or obese. Obesity is a well-established risk factor for degenerative spinal disease and lumbar disc herniation (22). Increased axial loading, chronic low-grade systemic inflammation, adipokine dysregulation, and altered spinal biomechanics have all been implicated in obesity-associated disc degeneration (22, 23).

Epidemiological evidence further supports a significant association between elevated BMI, chronic low back pain, and lumbar disc herniation (23). Interestingly, spondylolisthesis was the most frequent MRI finding in our cohort, exceeding disc protrusion and extrusion patterns typically reported in comparable studies. This discrepancy may be explained by the surgical nature of the study population, as all patients were candidates for discectomy and likely represented more advanced degenerative or instability-related pathology (24). Additionally, the higher prevalence of obesity may have contributed to increased mechanical instability and vertebral slippage (22). Variability in MRI classification systems and overlapping degenerative features may also account for inter-study differences (24).

The detection of anaerobic growth in this study was based solely on turbidity in thioglycollate broth and should therefore be interpreted as presumptive rather than definitive identification. The absence of dedicated anaerobic culture conditions limited further characterization at the genus and species levels. The present study evaluated the microbiological profile and antimicrobial resistance patterns of bacteria isolated from extruded lumbar disc specimens obtained during lumbar discectomy. A subset of disc samples demonstrated bacterial growth, predominantly involving low-virulence anaerobic and skin-associated organisms, while approximately two-thirds of specimens were culture-negative. Anaerobic bacteria, CoNS, and diphtheroid organisms were the most frequently isolated pathogens. In addition, MDR phenotypes, including MRSA and CRPA, were identified among several isolates. The role of bacterial colonization in intervertebral disc degeneration remains controversial. Since the seminal report by Stirling et al. describing Cutibacterium acnes DNA in herniated disc tissue, increasing evidence has suggested a possible association between low-grade bacterial infection and chronic disc inflammation (25).

Proposed mechanisms include hematogenous bacterial dissemination into disrupted disc tissue, biofilm formation within the avascular disc environment, and activation of pro-inflammatory cytokines that promote matrix degradation and nociceptive sensitization (26, 27). However, interpretation of positive microbiological findings remains challenging. Several studies have argued that organisms such as CoNS and diphtheroids may represent perioperative contamination or normal skin flora rather than true intradiscal infection (28). The predominance of low-virulence organisms in the present study supports this hypothesis. Nevertheless, emerging evidence suggests that such bacteria may persist in biofilm form and contribute to chronic subclinical inflammation without overt infection (29, 30).

Distinguishing contamination from true intradiscal infection remains a major methodological limitation in this field (28). The relatively high proportion of culture-negative samples in our study may be explained by multiple factors. The avascular nature of intervertebral disc tissue limits bacterial load and reduces culture sensitivity. Conventional culture methods may fail to detect fastidious, slow-growing, or biofilm-associated microorganisms. In addition, prior or unreported antibiotic exposure may suppress bacterial growth. Finally, technical limitations in anaerobic culture techniques may have led to underestimation of anaerobic organisms (31). Although detected at low frequency, the isolation of Escherichia coli and Pseudomonas aeruginosa is clinically relevant, as these organisms are not typical skin flora and may reflect opportunistic colonization, healthcare-associated contamination, or true infection in selected cases (32).

Their presence warrants cautious interpretation, particularly in patients with prior hospitalization or spinal interventions. A notable finding of this study was the detection of multidrug-resistant organisms. The identification of carbapenem-resistant *P. aeruginosa* (CRPA) is particularly concerning, reflecting the global rise of antimicrobial resistance in healthcare-associated pathogens (33).

Similarly, reduced susceptibility to beta-lactams among CoNS and E. coli isolates, along with cefoxitin resistance in approximately 40% of *S. aureus* isolates (suggesting MRSA), highlights the increasing burden of MDR organisms in surgical settings (33, 34).

The clinical significance of these findings should be interpreted cautiously. While the pathogenic role of isolated organisms remains uncertain, the presence of

MDR bacteria may still be relevant for perioperative infection control, empirical antibiotic selection, and postoperative surveillance. These findings further underscore the importance of antimicrobial stewardship and strict aseptic surgical protocols in spinal procedures (33, 34).

Nevertheless, several limitations should be acknowledged. Although strict aseptic procedures were followed during specimen collection and processing, contamination by skin commensals, particularly coagulase-negative staphylococci (CoNS) and diphtheroids, cannot be entirely ruled out. Furthermore, more than 60% of the specimens yielded negative culture results, which may reflect a low microbial burden within disc tissue, prior antibiotic exposure, limitations of conventional culture techniques, or the presence of fastidious microorganisms that are difficult to recover under routine laboratory conditions. The lack of advanced molecular diagnostic tools and optimized anaerobic culture systems may have further reduced the detection of low-abundance or difficult-to-cultivate organisms. Therefore, the findings of this study should be interpreted with caution, and future investigations incorporating molecular methods and comprehensive anaerobic culture techniques are needed to better characterize the microbial profile of lumbar disc tissue

Conclusion

Overall, the findings of this study support the hypothesis that bacterial colonization may be present in a subset of extruded lumbar disc specimens; however, the biological and clinical significance of these microorganisms remains uncertain. Future large-scale multicenter studies integrating advanced molecular diagnostics, quantitative microbiome analysis, standardized contamination-control protocols, and longitudinal clinical follow-up are necessary to clarify whether bacterial involvement represents true pathogenic contribution, transient colonization, or perioperative contamination in lumbar disc disease.

Declaration

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request

Conflict of Interest

There are no conflicts or contradictions in this collaboration.

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No funding was received for this study.

Authors' contribution

All authors were participated in the concept and design, analysis and interpretation, data collection, writing the article, critical revision, final approval, statistical analysis, overall responsibility.

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Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Babol University of Medical Sciences under the ethics approval code IR.MUBABOL.REC.1403.135 and research tracking code 724134830.

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