

Article

Diagnostic Performance of [^{99m}Tc]Tc-UBI 29-41 SPECT/CT with a Standardized Semiquantitative Target-to-Liver Ratio Analysis in Four Difficult-to-Diagnose Bacterial Infection Conditions: A Prospective Diagnostic Accuracy Study

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Abstract

Objectives: To assess whether [^{99m}Tc]Tc-UBI 29-41 SPECT/CT combined with a standardized target-to-liver ratio can reliably support clinical decision-making in patients with difficult-to-diagnose bacterial infections and to establish a reproducible diagnostic threshold applicable across four infectious scenarios. **Methods:** This prospective diagnostic accuracy study, conducted following STARD guidelines, included 156 consecutive patients (346 lesions) with clinical suspicion of infected arthroplasty, spondylodiscitis, osteomyelitis, or fever of unknown origin. Target-to-liver total count ratios were calculated from SPECT/CT images. Diagnostic performance was assessed using bootstrap-resampled ROC analysis, Fagan nomograms, and penalized logistic regression. **Results:** All four conditions showed good discriminatory capacity (AUC: 0.965–0.979). At a cutoff ratio ≥ 1.5 , specificities exceeded 95% and negative predictive values were $\geq 97.8\%$ across all subgroups. Post-test probability after a negative scan decreased to 2–3% in every condition, consistent with clinically meaningful rule-out capability across all four scenarios. Penalized logistic regression confirmed significant associations between elevated ratios and confirmed infection (OR: 22.02–245.53; all $p \leq 0.003$). **Conclusions:** [^{99m}Tc]Tc-UBI 29-41 SPECT/CT with target-to-liver ratio analysis provides clinically meaningful diagnostic support across four distinct infectious scenarios, with particular strength as a rule-out test. A threshold of ≥ 1.5 offers a standardized, potentially reproducible criterion that may guide clinical decisions and reduce reliance on invasive confirmatory procedures. Prospective multicentre validation is warranted to establish this bacteria-specific approach as a practical complement to existing diagnostic algorithms.

Keywords: [^{99m}Tc]Tc-UBI 29-41; SPECT/CT; semiquantitative analysis; diagnostic accuracy; musculoskeletal infection; bacteria-specific imaging



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1. Introduction

Bacterial infections remain a major cause of morbidity and mortality worldwide, and their diagnosis is often difficult when conventional clinical and laboratory findings are inconclusive, leading to delays in antimicrobial therapy [1–3]. With antimicrobial resistance projected to become one of the leading causes of global mortality by 2050, the development of diagnostic tools that can rapidly localize infectious foci is becoming increasingly urgent [1]. The standard diagnostic workup relies on clinical assessment, inflammatory biomarkers, imaging, and microbiological identification [2]. However, conventional radiological imaging performs poorly when morphological changes are absent, when metallic implants are present, or when postsurgical anatomy is distorted [3]. Nuclear medicine addresses some of these gaps using radiolabelled probes that target physiological changes at the infection site, allowing earlier detection and treatment monitoring [4]. Nevertheless, despite clear descriptions of the ideal characteristics for infection-imaging radiopharmaceuticals, currently available agents still have important limitations [5,6], compounded by the lack of standardized acquisition and interpretation protocols. Radiolabelled leukocytes [^{111}In]In-Oxime, [$^{99\text{m}}\text{Tc}$]Tc-HMPAO and ^{18}F -FDG cannot reliably distinguish bacterial infection from sterile inflammation, as all accumulate at sites of increased metabolic activity regardless of the presence or absence of viable microorganisms [7,8]. ^{18}F -FDG reaches infectious foci through upregulated glucose transport and enhanced cellular metabolism, a process that occurs in both bacterial and sterile inflammatory environments. Current guidelines support its use in fever of unknown origin (diagnostic yield: 50–60%) [9], diabetic foot infections (sensitivity: 89%; specificity: 92%) [10], and spondylodiscitis (sensitivity: 85%; specificity: 92%) [11]. Radiolabelled leukocyte scintigraphy with [$^{99\text{m}}\text{Tc}$] or [^{111}In], although considered the reference standard for periprosthetic and musculoskeletal infections [3], remains a resource-intensive procedure that requires a dedicated radiopharmaceutical infrastructure for ex vivo cell labelling and autologous reinjection [12].

This clinical need for pathogen-directed agents, capable of localizing bacteria directly at the infection site independent of the host inflammatory response, has driven research into antimicrobial peptide-based radiopharmaceuticals. Early work showed that $^{99\text{m}}\text{Tc}$ -labeled human neutrophil peptide-1 (HNP-1), an α -defensin, accumulated at sites of experimental *S. aureus* infection [13]. A subsequent screening study evaluated three ubiquicidin-derived fragments—UBI 29-41, UBI 18-35, and UBI 31-38—together with the lactoferrin fragment hLF 1-11 and defensins, all labeled with $^{99\text{m}}\text{Tc}$; each peptide accumulated significantly more in infected tissues than in sterile inflammatory lesions [14], as reviewed by Lupetti et al. [15]. Among these candidates, the cationic synthetic fragment ubiquicidin (UBI 29-41) progressed furthest towards clinical application, largely because of its favorable infection-to-inflammation ratio and pharmacokinetic profile. It has been successfully labelled with ^{68}Ga for PET and $^{99\text{m}}\text{Tc}$ for SPECT imaging [16]. UBI 29-41 carries a net positive charge of approximately +5 at physiological pH, which drives its preferential binding to bacterial and fungal cell membranes: prokaryotic membranes expose anionic phospholipids (phosphatidylglycerol, cardiolipin) on their outer layer, whereas mammalian membranes present zwitterionic phospholipids externally and contain cholesterol that limits peptide insertion [17,18]. This charge-based selectivity is what allows UBI 29-41 to concentrate at sites where viable bacteria are present while showing minimal binding to host tissues—the pharmacological basis for its progression to clinical use [19]. Evidence suggests that it can be used to monitor antimicrobial treatment response noninvasively [20].

A practical advantage of [$^{99\text{m}}\text{Tc}$]Tc-UBI 29-41 over radiolabelled leukocyte scintigraphy is that it can be used in leukopenic and febrile neutropenic patients, in whom ex vivo white blood cell labelling is contraindicated or simply not feasible due to insufficient circulating

leukocytes. As UBI 29-41 directly targets bacterial membranes without depending on the host immune response, UBI provides a diagnostic alternative in immunocompromised individuals [17]. In vivo and preclinical data have also shown that [^{99m}Tc]Tc-UBI 29-41 accumulation is correlated with the number of viable bacteria at the infection site, with an inverse relationship between tracer uptake and antibiotic dose, findings that support its potential as a biomarker for monitoring treatment response [19–21]. However, it has certain limitations: it cannot be used to discriminate between bacterial species, its binding is restricted to organisms with exposed cell membranes (excluding intracellular or phagocytized bacteria), and the minimum detectable bacterial burden has been estimated at 10^3 – 10^4 colony-forming units [20,22].

Clinical studies using [^{99m}Tc]Tc-UBI 29-41 have shown consistently good diagnostic performance for various infectious indications. In a pooled analysis, Ostovar et al. reported an overall accuracy of 93.7% for musculoskeletal, prosthetic, and soft tissue infections [23]. Sathekge et al. reported sensitivities and specificities of 99% and 94.5%, respectively, for osteomyelitis evaluated with SPECT/CT [24], and Sepúlveda-Méndez et al. reported 96.2% accuracy in patients with fever of unknown origin [25]. Despite these results, the evidence base remains limited by small cohort sizes, variability in administered activities and acquisition timing, and the absence of standardized interpretation criteria. Furthermore, existing studies lack a validated, reproducible semiquantitative threshold across indications. These factors introduce reader-dependent variability and potential bias and leave an unresolved need for a validated, reproducible approach to distinguish bacterial infection from sterile inflammation [26].

This study was designed to evaluate whether difficult-to-diagnose infectious foci can be reliably identified using [^{99m}Tc]Tc-UBI 29-41 SPECT/CT combined with a semiquantitative analysis based on target-to-liver total count ratios and to determine which ratio threshold best predicts infection. As a secondary objective, this study sought to establish potential cut-off values that could support diagnostic decision-making and reduce the variability inherent in visual scan interpretation.

2. Materials and Methods

2.1. Study Design and Ethical Considerations

This single-center diagnostic accuracy study was based on the retrospective analysis of clinical and imaging data that had been prospectively collected during routine patient care, and was conducted in accordance with the Standards for Reporting of Diagnostic Accuracy (STARD) guidelines for transparent reporting [27]. Ethical approval was granted by the institutional investigation committee (registration number: CEIFUS 004-26). Between November 2024 and December 2025, all [^{99m}Tc]Tc-UBI 29-41 SPECT/CT examinations were performed as part of routine clinical care for patients referred with suspected bacterial infection; clinical and imaging data were recorded in a departmental database as standard documentation practice. In September 2025, the research team began developing the formal study protocol in collaboration with the institutional epidemiology department. The protocol was submitted to the ethics committee in December 2025, and approval was granted on 9 January 2026. No research-related data analysis was initiated until after ethical approval was obtained. Patient enrollment for the purpose of this study was closed at the time of approval. The study was performed in accordance with the principles of the 1964 Declaration of Helsinki and its subsequent amendments, as well as Good Clinical Practice guidelines.

2.2. Study Population

A total of 247 consecutive patients were clinically evaluated between November 2024 and December 2025 via non-probabilistic, consecutive sampling. Patients were referred to the nuclear medicine department with suspected difficult-to-diagnose bacterial infection characterized by positive inflammatory markers, clinical signs of infection, and the absence of a clearly identifiable infectious focus. Inclusion criteria comprised all patients meeting these clinical criteria who underwent [^{99m}Tc]Tc-UBI 29-41 imaging. Exclusion criteria comprised patients in whom infection could neither be confirmed nor ruled out by the reference standard, including biopsy, microbiological culture, cross-sectional imaging (computed tomography [CT] or magnetic resonance imaging [MRI]), or clinical follow-up. The sample size was calculated using a single-sample ROC curve analysis, following the framework described by Hanley and McNeil. Under a null hypothesis AUC of 0.60 and an expected AUC of 0.70 under the alternative hypothesis, a two-sided test was conducted with a 5% significance level and 80% statistical power. Under these assumptions, a minimum of 240 lesions was needed, and an equal proportion of positive and negative cases (1:1) was assumed. After adjusting for 10% attrition, the final estimated sample size was 268 lesions. After application of the inclusion and exclusion criteria, 156 patients were included in the final analysis, contributing 346 lesions for the lesion-based analytical approach. Patients were categorized into five diagnostic subgroups according to the pre-imaging clinical indication: infected arthroplasty, spondylodiscitis, osteomyelitis, fever of unknown origin, and vascular prosthesis infection. However, due to the limited sample size of the vascular prosthesis infection subgroup ($n = 16$; 4.62%), it was not possible to perform a reliable diagnostic accuracy analysis under the proposed semiquantitative methodology for this condition. Consequently, the subgroup-specific analyses of diagnostic performance, logistic regression, and post-test probability estimation were restricted to the remaining four clinical conditions.

Patient selection process is summarized in Figure 1.

2.3. SPECT/CT Imaging Protocol

All patients underwent whole-body planar imaging 50 min following the intravenous administration of 400 MBq of [^{99m}Tc]Tc-UBI 29-41. The imaging protocol consisted of whole-body planar acquisition combined with SPECT/CT of the thorax, abdomen, and/or extremities according to the clinical indication. Data acquisition was performed on a dedicated hybrid SPECT/CT system (Discovery NM/CT 860, GE Healthcare, Milwaukee, WI, USA) equipped with a low-energy high-resolution (LEHR) collimator. The energy peak was centred at 140 keV with a symmetric 15% window. The SPECT acquisition parameters included a 128×128 matrix with 128 projections at 30 s per step. Image reconstruction was performed using three-dimensional ordered subset expectation maximization (3D-OSEM; 4 iterations, 8 subsets). Low-dose CT acquisition was performed using the integrated 8-slice CT component (Revolution ACTs) for anatomical correlation and attenuation correction (140 kV, 20 mAs, rotation speed 0.6 s, collimation 8×0.75 mm, and a slice thickness of 1.25 mm). Both CT attenuation-corrected and non-corrected SPECT images were transferred in DICOM format to a Xeleris 4.1 workstation (GE Healthcare) for reconstruction and fusion. Images were evaluated in the coronal, transaxial, and sagittal planes.

STARD 2015 Flow Diagram: Participant Flow Through the Study

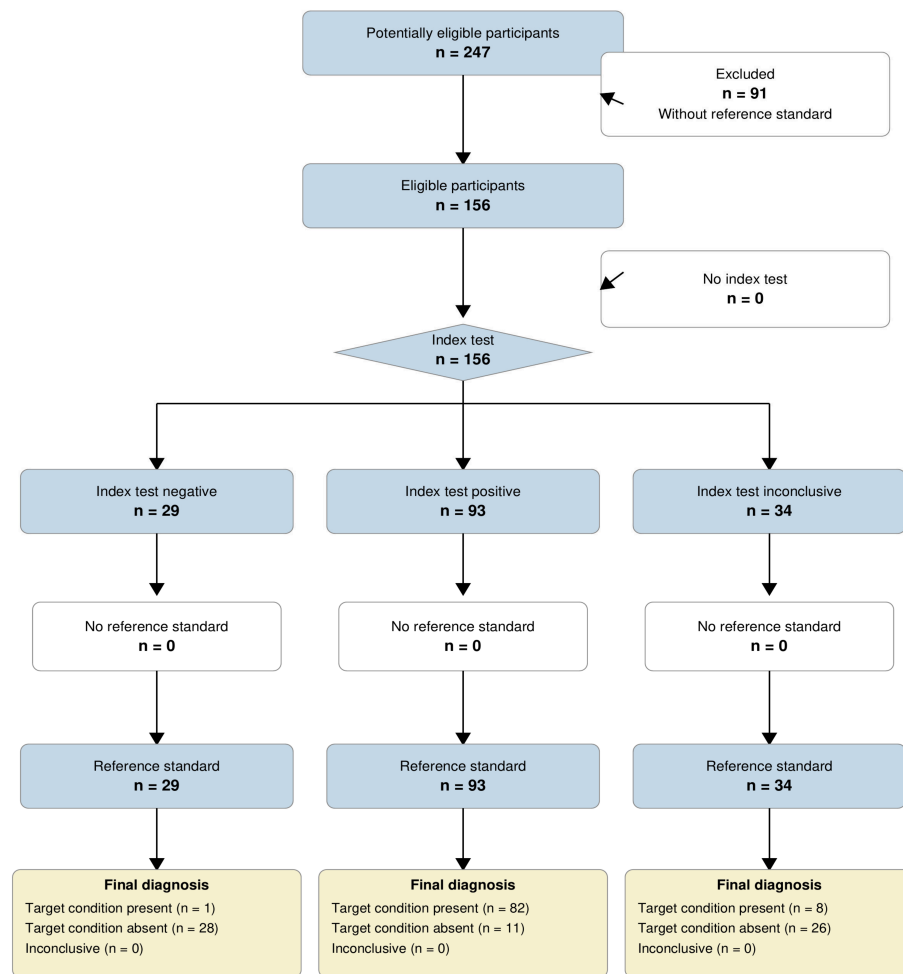


Figure 1. STARD 2015 flow diagram illustrating the flow of participants through the study. Of 247 potentially eligible patients, 91 were excluded due to absence of reference standard confirmation. The remaining 156 patients underwent [^{99m}Tc]Tc-UBI 29-41 SPECT/CT (index test) and were classified into three groups based on target-to-liver ratios: negative ($n = 29$), positive ($n = 93$), and inconclusive ($n = 34$). All patients had their final diagnoses confirmed by the reference standard.

2.4. Semiquantitative Analysis

For semiquantitative analysis, total counts were measured at the suspected lesion site (target region), over the liver (reference organ), and over a vertebral body distant from the region of clinical interest (normal reference region). Equal-sized regions of interest (ROIs) were duplicated for count measurement. The following ratios were calculated: *Target ratio* = total counts at the suspected lesion/total counts at the liver.

Normal zone ratio = total counts at the reference vertebra/total counts at the liver. As the inclusion of healthy control subjects was ethically precluded, the unit of analysis was the ratio, with each target ratio being paired with a corresponding normal zone ratio obtained from the same patient. The liver was selected as the reference organ based on: (a) its consistent hepatic uptake during the 1–2 h acquisition window, as demonstrated in the human biodistribution study by Welling et al. (liver uptake $5.43\% \pm 0.76\%$ at 30 min, $3.17\% \pm 0.25\%$ at 120 min) [28]; and (b) its established use as the visual reference in prior UBI 29-41 studies, where the scoring system classifies uptake relative to liver activity [24]. All measurements were performed by two independent readers and cross-validated by a nuclear medicine physician with more than 20 years of experience in SPECT/CT interpretation.

2.5. Diagnostic Reference Standard

A definitive diagnosis of infection was established using a composite reference standard appropriate for clinical context, including biopsy with histopathological examination, intraoperative findings, culture and microbiological analysis, advanced cross-sectional imaging (MRI, CT), and/or clinical outcome, defined as documented improvement following targeted antibiotic therapy.

2.6. Statistical Analysis

Descriptive statistics were performed to characterize the study population; qualitative variables were summarized as absolute and relative frequencies, while continuous variables were reported as medians and interquartile ranges (IQR) due to the rejection of the assumption of normality. Diagnostic agreement was assessed using Cohen's kappa coefficient with 95% confidence intervals. Agreement was evaluated between the clinician's presumptive diagnosis, the diagnostic classification based on the calculated ratios, and the clinically confirmed diagnosis based on the reference standard (diagnostic post-test). To improve the robustness of the agreement estimates, bootstrap resampling was performed with 1000 replicates. The relationship between the ratios and the clinical diagnosis was evaluated using a Firth logistic regression model, along with bootstrap resampling performed at the patient level to preserve within-subject correlation (1000 iterations) and estimate the odds ratio (OR) and its corresponding confidence interval. Furthermore, the diagnostic performance of the ratio was evaluated separately for each clinical condition (arthroplasty, spondylodiscitis, osteomyelitis, and fever of unknown origin). ROC curves were constructed, and the area under the curve (AUC) was estimated using bootstrap resampling (1000 iterations) to obtain stable confidence intervals. As a single patient could have multiple lesions, the logistic regression models and bootstrap estimates incorporated patient-level clustering to account for within-subject correlation, using robust standard errors. An optimal cut-off point of 1.5 was proposed for the ratio based on diagnostic accuracy criteria, with a specificity greater than 95%. At this threshold, the sensitivity, specificity, positive and negative likelihood ratios (LR+ and LR−), positive predictive value (PPV), and negative predictive value (NPV) were calculated with corresponding 95% confidence intervals. Post-test probabilities were estimated using likelihood ratios and Fagan nomograms. Kernel density estimation was used to visualize ratio distributions in infected versus noninfected lesions. All analyses were performed using Stata 17 MP (StataCorp LLC, College Station, TX, USA), with a two-sided $p < 0.05$ considered significant.

3. Results

The patient demographics and clinical characteristics are summarized in Table 1. A total of 156 patients with 346 lesions were included in the lesion-based analysis. The cohort had a slight male predominance ($n = 85$; 54.49%), with a median age of 61 years (IQR: 43–72.5). The most frequent clinical indication was osteomyelitis ($n = 52$; 15.03%), followed by fever of unknown origin ($n = 39$; 11.27%), infected arthroplasty ($n = 35$; 10.12%), spondylodiscitis ($n = 31$; 8.96%), and vascular prosthesis infection ($n = 16$; 4.62%). Half of the analysed lesions ($n = 173$) corresponded to anatomical regions without pathology and served as internal controls. As the vascular prosthesis subgroup had too few confirmed cases for reliable diagnostic accuracy estimation using the ratio-based methodology, the subsequent subgroup analyses were limited to the four remaining conditions (Tables 2 and 3, Figures 2–4).

Across the 346 lesions, liver total counts had a mean of 488 ± 204 (median 523; range 15–1007). Counts at suspected infection sites averaged 486 ± 232 in noninfected lesions and 805 ± 420 in confirmed infections. Normal reference vertebral body counts were 345 ± 167 . The inter-patient coefficient of variation (CV) of liver counts was 41.7%. Because our

analysis was built on a paired intra-patient design—each target-to-liver ratio paired with a normal reference ratio from the same patient in the same acquisition—the intra-patient CV of liver counts was below 5% in 92.3% of patients and below 10% in 96%.

Table 1. Demographic and clinical characteristics of the study population.

Characteristics	Total (<i>n</i> = 156)
Sex, <i>n</i> (%)	
Female	71 (45.51%)
Male	85 (54.49%)
Age, years, median (IQR)	61.00 (43.00–72.50)
Age Group, <i>n</i> (%)	
13–18 years	3 (1.92%)
18–40 years	24 (15.38%)
40–60 years	46 (29.49%)
>60 years	83 (53.21%)
Pre-Imaging Clinical Indication, <i>n</i> (%) ^a	
Infected arthroplasty	35 (10.12%)
Spondylodiscitis	31 (8.96%)
Fever of unknown origin	39 (11.27%)
Osteomyelitis	52 (15.03%)
Vascular prosthesis infection	16 (4.62%)
Lesions without pathology (internal controls)	173 (50.00%)

Data are presented as median (IQR) for continuous variables and *n* (%) for categorical variables. ^a Based on 346 lesions analyzed.

3.1. Receiver Operating Characteristic Analysis

Figure 2 shows the ROC curves for each clinical condition, constructed with bootstrap resampling (1000 iterations). All four subgroups showed good overall discriminatory capacity. Infected arthroplasty had a bootstrap AUC of 0.949 (95% CI: 0.909–0.988), spondylodiscitis 0.960 (95% CI: 0.913–1.000), osteomyelitis 0.967 (95% CI: 0.938–0.995), and fever of unknown origin 0.969 (95% CI: 0.938–0.998). All four curves showed a characteristic plateau at high sensitivity levels, indicating that the semiquantitative ratio maintained good sensitivity across a wide range of specificity thresholds.

3.2. Distribution of Uptake Ratios by Clinical Condition

Figure 3 shows the kernel density distributions of target-to-liver uptake ratios for infected versus noninfected lesions (internal controls) across the four clinical conditions. The vertical dashed line at a ratio = 1.5 indicates the proposed diagnostic cut-off. In all subgroups, the noninfected population showed a narrow, right-skewed distribution centred below 1.0, whereas the distributions of infected lesions shifted to the right, with peaks above the 1.5 cut-off. For infected arthroplasty, the infected distribution was broad and centred between 1.5 and 2.5, with minimal overlap near the cut-off. Spondylodiscitis showed moderate overlap in the cut-off region, which is consistent with the wider confidence interval observed for sensitivity in this condition, although most of the infected distribution extended well beyond 1.5. In patients with osteomyelitis, the distribution of infections was centred slightly above 1.5, reaching ratios above 3.5 and showing relatively clear separation from the noninfected population. Fever of unknown origin showed the most pronounced separation, with the infection curve centred at approximately 2.0–2.5 and extending above 4.5, which is consistent with the high diagnostic performance reported in Table 2. In all conditions, overlap between the two distributions was concentrated in the 1.0–1.5 range, the zone of greatest diagnostic uncertainty, which further supported a ratio ≥ 1.5 as the optimal cut-off.

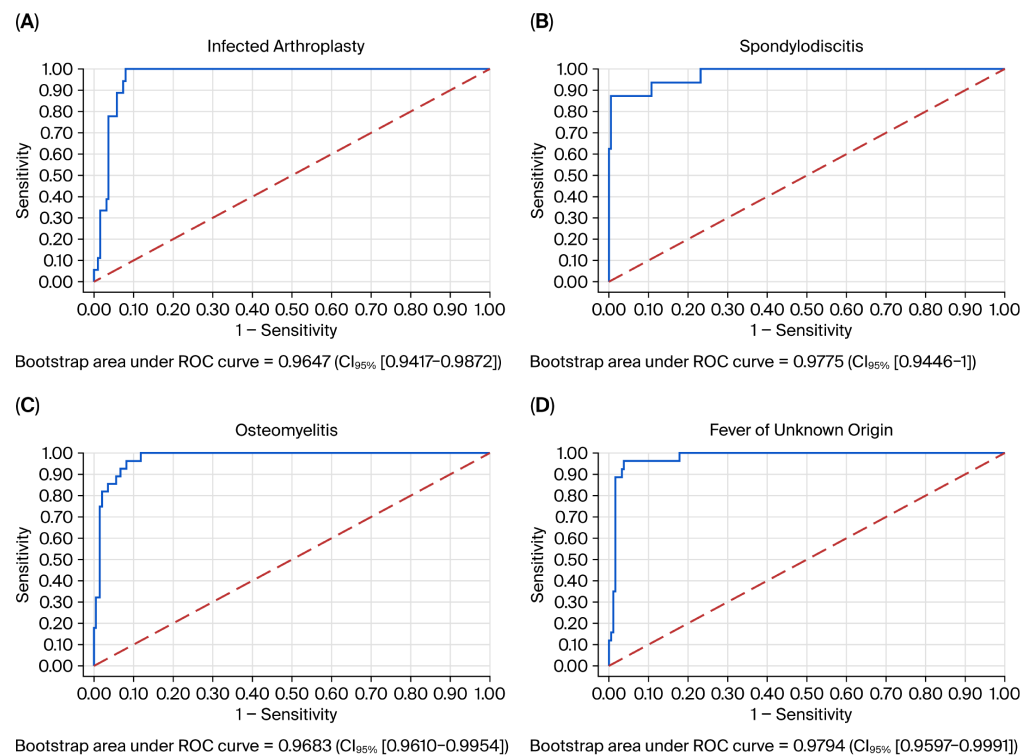


Figure 2. Receiver operating characteristic (ROC) curves of [^{99m}Tc]Tc-UBI 29-41 SPECT/CT target-to-liver uptake ratios for the diagnosis of (A) infected arthroplasty, (B) spondylodiscitis, (C) osteomyelitis, and (D) fever of unknown origin. The area under the curve (AUC) was estimated by bootstrap resampling (1000 iterations) with 95% confidence intervals. The dashed diagonal line represents the reference line of no diagnostic discrimination (AUC = 0.5).

3.3. Diagnostic Performance by Clinical Condition

Table 2 presents the diagnostic accuracy metrics for the four conditions at a cut-off ratio ≥ 1.5 . ROC analysis with bootstrap resampling revealed good discrimination across all subgroups, with AUC values ranging from 0.965 (infected arthroplasty) to 0.979 (osteomyelitis). In infected arthroplasty ($n = 207$ lesions), the AUC was 0.965 (95% CI: 0.942–0.988), sensitivity was 77.8% (95% CI: 52.4–93.6%), and specificity was 95.2% (95% CI: 91.2–97.8%). For spondylodiscitis ($n = 202$ lesions), the AUC was 0.977 (95% CI: 0.945–1.000), sensitivity was 87.5% (95% CI: 61.7–98.4%), and specificity was the highest among all subgroups at 98.9% (95% CI: 96.2–99.9%). Fever of unknown origin ($n = 222$ lesions) had an AUC of 0.978 (95% CI: 0.961–0.995), sensitivity of 85.7% (95% CI: 67.3–96.0%), and specificity of 95.9% (95% CI: 92.0–98.2%). The NPV of 97.9% (95% CI: 94.7–99.4%) highlights the value of a negative scan for ruling out infectious aetiologies. Osteomyelitis ($n = 211$ lesions) had the highest AUC (0.979; 95% CI: 0.960–0.999), with a sensitivity of 88.5% (95% CI: 69.8–97.6%) and a specificity of 96.8% (95% CI: 93.1–98.8%), and the highest NPV among all conditions was 98.4% (95% CI: 95.3–99.7%).

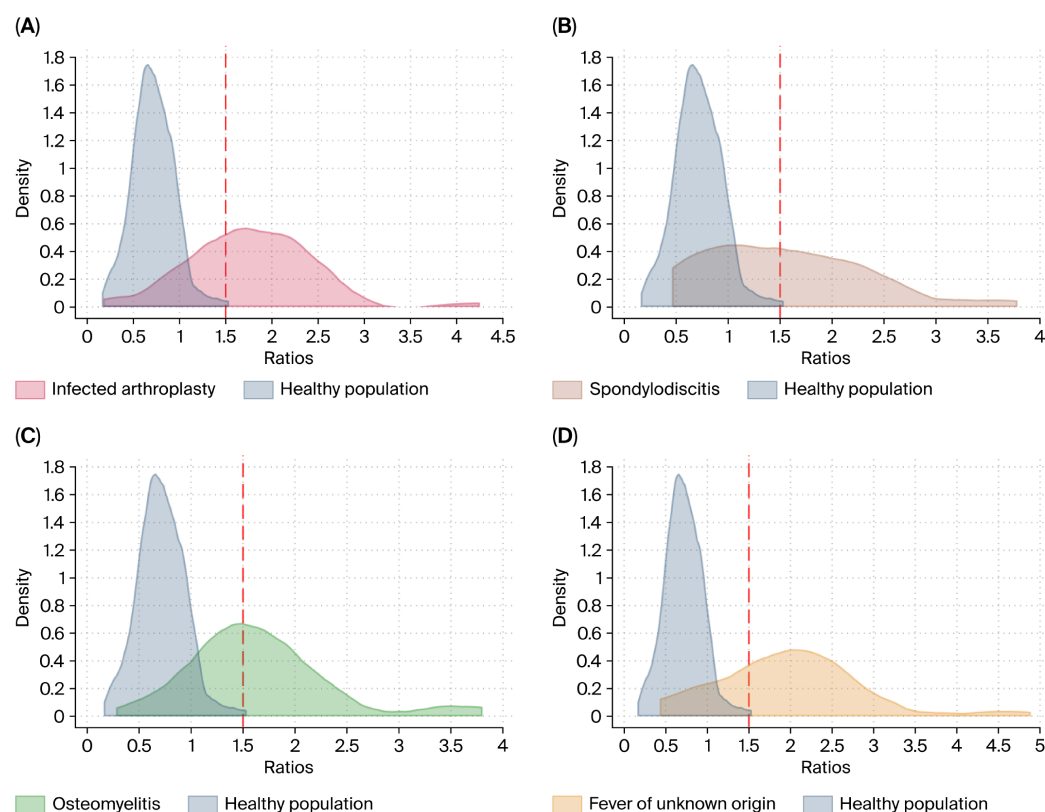


Figure 3. Kernel density plots of [^{99m}Tc]Tc-UBI 29-41 SPECT/CT target-to-liver uptake ratios for lesions with confirmed infection (colored distributions) versus lesions without pathology (blue-gray distributions, internal controls) across the four clinical conditions: (A) infected arthroplasty, (B) spondylodiscitis, (C) osteomyelitis, and (D) fever of unknown origin. The vertical red dashed line indicates the proposed diagnostic cutoff (Ratio ≥ 1.5). The degree of separation between distributions reflects the discriminatory capacity of the semiquantitative ratio approach for each condition.

Table 2. Diagnostic performance of [^{99m}Tc]Tc-UBI 29-41 SPECT/CT by clinical condition.

Condition	<i>n</i> ^a	AUC ^b (95% CI)	Sensitivity, % (95% CI) ^c	Specificity, % (95% CI) ^c	LR+ (95% CI) ^c	LR− (95% CI) ^c	PPV, % (95% CI) ^c	NPV, % (95% CI) ^c
Infected arthroplasty	207	0.965 (0.942–0.988)	77.8 (52.4–93.6)	95.2 (91.2–97.8)	16.3 (8.24–32.4)	0.23 (0.10–0.55)	60.9 (38.5–80.3)	97.8 (94.5–99.4)
Spondylodiscitis	202	0.977 (0.945–1.000)	87.5 (61.7–98.4)	98.9 (96.2–99.9)	81.4 (20.3–327)	0.13 (0.03–0.46)	87.5 (61.7–98.4)	98.9 (96.2–99.9)
Fever of unknown origin	222	0.978 (0.961–0.995)	85.7 (67.3–96.0)	95.9 (92.0–98.2)	20.8 (10.4–41.7)	0.15 (0.06–0.37)	75.0 (56.6–88.5)	97.9 (94.7–99.4)
Osteomyelitis	211	0.979 (0.960–0.999)	88.5 (69.8–97.6)	96.8 (93.1–98.8)	27.3 (12.3–60.7)	0.12 (0.04–0.35)	79.3 (60.3–92.0)	98.4 (95.3–99.7)

^a *n* represents the number of lesions analyzed in each diagnostic subgroup versus lesions without findings (*n* = 137). ^b ROC area estimated by bootstrap (1000 repetitions). ^c 95% CI calculated by normal approximation. Sensitivity, specificity, and likelihood ratios obtained at the optimal cutoff (Ratio ≥ 1.5).

3.4. Post-Test Probability Analysis

Figure 4 shows the Fagan nomograms illustrating the shift from pre-test to post-test probability for each condition, based on the likelihood ratios at the cut-off of ≥ 1.5 . Specific values are detailed in the figure legend. In all conditions, a positive scan result produced a substantial increase in the post-test probability, whereas a negative result reduced the post-test probability to 2–3%, supporting the rule-out value of [^{99m}Tc]Tc-UBI 29-41 SPECT/CT.

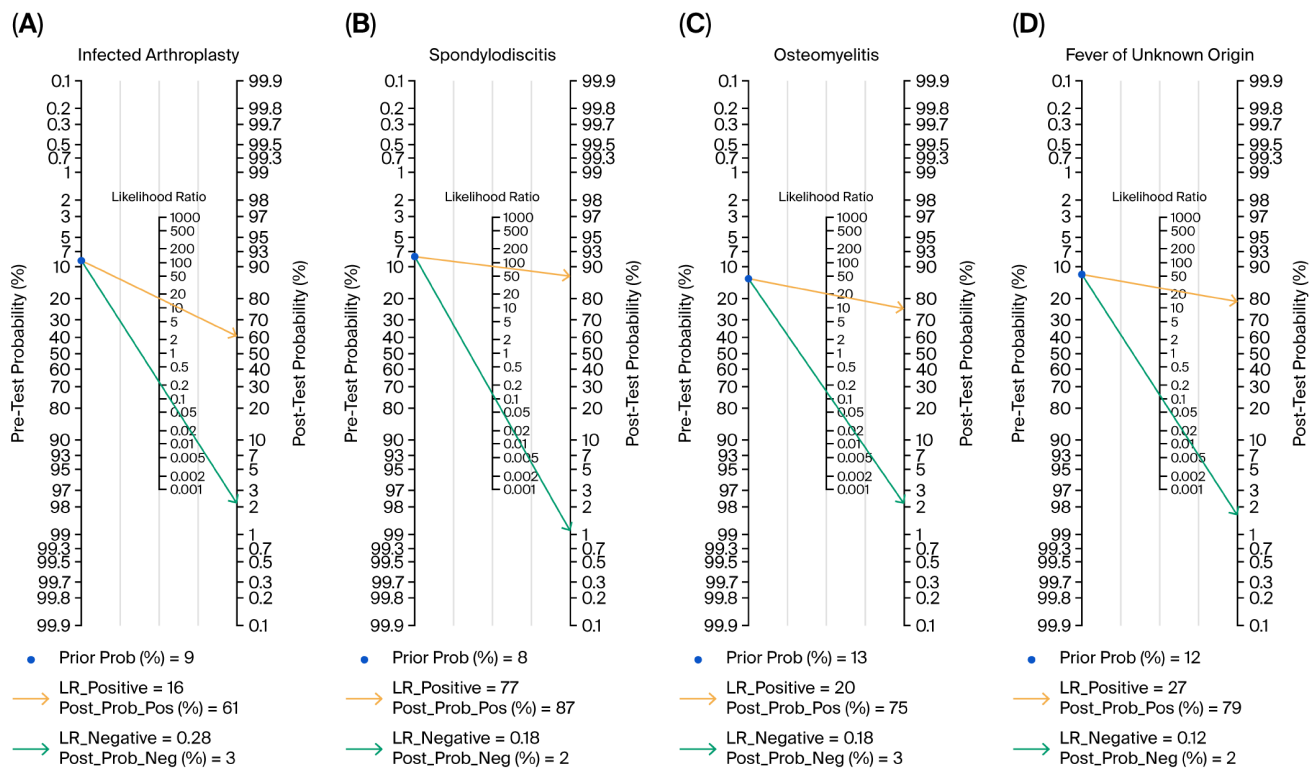


Figure 4. Fagan nomograms illustrating the transition from pre-test to post-test probability of infection for (A) infected arthroplasty (pre-test probability: 9%; LR+: 16; LR−: 0.28), (B) spondylodiscitis (pre-test probability: 8%; LR+: 77; LR−: 0.18), (C) osteomyelitis (pre-test probability: 13%; LR+: 20; LR−: 0.18), and (D) fever of unknown origin (pre-test probability: 12%; LR+: 27; LR−: 0.12). The blue solid line represents the probability shift for a positive test result; the green dashed line represents the probability shift for a negative test result. Post-test probabilities were calculated using the likelihood ratios derived from the optimal diagnostic cutoff (Ratio ≥ 1.5).

3.5. Logistic Regression Analysis

Table 3 shows the penalized logistic regression results. All four conditions showed statistically significant associations between elevated target-to-liver ratios and confirmed bacterial infection. The OR ranged from 22.02 (95% CI: 4.83–100.40; $p < 0.001$) for infected arthroplasty to 245.53 (95% CI: 3.04–19,769; $p < 0.001$) for spondylodiscitis. Osteomyelitis had an OR of 137.35 (95% CI: 14.52–1298; $p < 0.001$), and fever of unknown origin had an OR of 52.25 (95% CI: 3.85–708.28; $p = 0.003$).

Table 3. Penalized logistic regression analysis of [^{99m}Tc]-UBI 29-41 uptake ratios by clinical condition.

Diagnosis	<i>n</i>	OR (95% CI)	<i>p</i> -Value
Infected arthroplasty	207	22.02 (4.83–100.40)	<0.001
Spondylodiscitis	202	245.53 (3.04–19,769)	<0.001
Osteomyelitis	222	137.35 (14.52–1298)	<0.001
Fever of unknown origin	211	52.25 (3.85–708.28)	0.003

OR, odds ratio; CI, confidence interval. Penalized logistic regression (Firth's method) with bootstrap resampling (1000 iterations). Patient-level clustering applied.

3.6. Illustrative Clinical Cases

Figures 5 and 6 present two illustrative clinical cases highlighting the diagnostic utility of [^{99m}Tc] Tc-UBI 29-41 SPECT/CT. In the first case, a patient with fever of unknown origin demonstrated focal hepatic tracer uptake, consistent with a pyogenic liver abscess subsequently confirmed by clinical and microbiological findings. The second case corresponds

to a patient with persistent low back pain following lumbar instrumentation, in whom the image UBI results, was compatible with bacterial infection associated with spondylodiscitis.

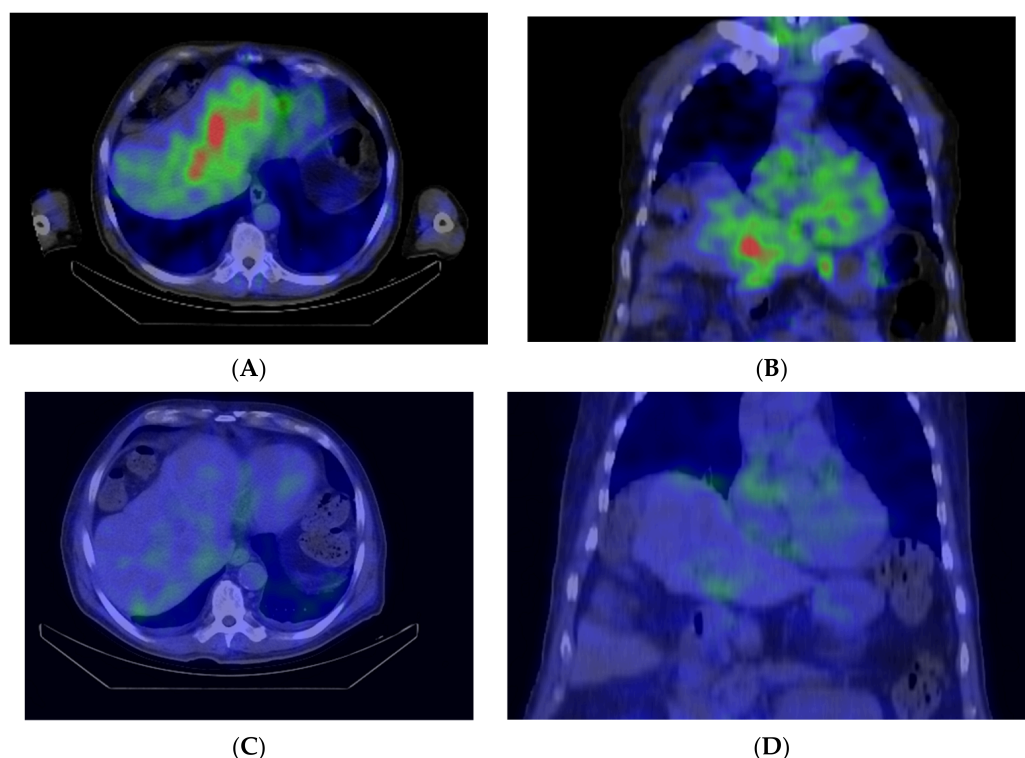


Figure 5. Fever of unknown origin with positive inflammatory markers and negative conventional imaging. Upper row: initial [^{99m}Tc]Tc-UBI 29-41 SPECT/CT in (A) axial and (B) coronal projections showing intense focal uptake in hepatic segment VIII (target-to-liver ratio: 3.8), consistent with hepatic abscess. Lower row: follow-up SPECT/CT 6 weeks after initiation of antibiotic therapy in (C) axial and (D) coronal projections demonstrating resolution of abnormal uptake with a normalized ratio of 1.0. Final diagnosis was confirmed by a positive immunoarray for *Shigella* intestinal infection with secondary hepatic involvement.

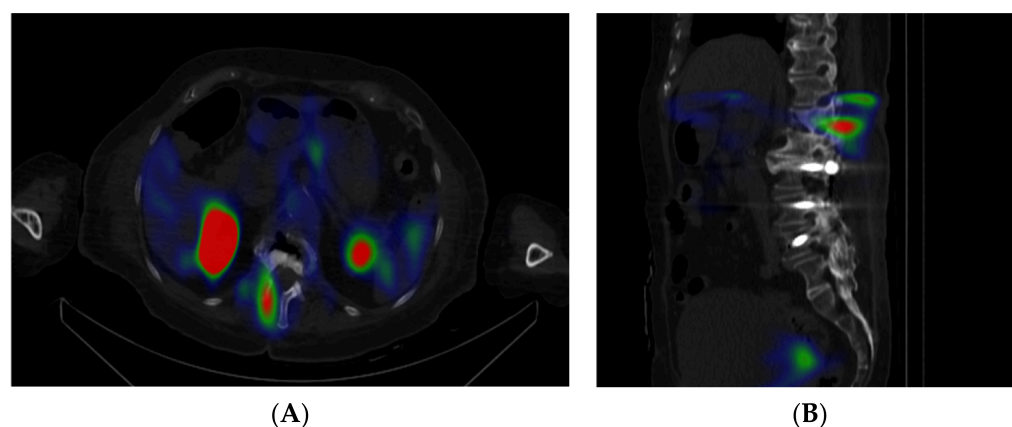


Figure 6. Postoperative lumbar instrumentation with persistent pain 3 months after surgery. MRI demonstrated inflammatory changes that could not be reliably distinguished from expected postoperative findings. In the absence of PET availability, [^{99m}Tc]Tc-UBI 29-41 SPECT/CT was performed. (A) Axial and (B) sagittal images showing intense focal uptake in the right posterior elements and right epidural space at L2, extending to the posterior soft tissues at the L1 level, consistent with active infection. This case exemplifies the added diagnostic value of [^{99m}Tc]Tc-UBI 29-41 in centers without access to PET/CT, where conventional imaging alone may be insufficient to differentiate postoperative inflammation from infection.

4. Discussion

This prospective diagnostic accuracy study represents the largest prospective cohort evaluating [^{99m}Tc]Tc-UBI 29-41 SPECT/CT with a standardized semiquantitative approach and is the first study to propose a specific target-to-liver ratio cut-off applicable across four different infectious conditions: infected arthroplasty, spondylodiscitis, osteomyelitis, and fever of unknown origin. The semiquantitative method showed good discriminatory capacity in all four subgroups (AUC: 0.965–0.979; specificities >95%), and a cut-off ratio ≥ 1.5 consistently yielded high negative predictive values ($\geq 97.8\%$), with post-test probability reduction to 2–3% after a negative scan. Penalized logistic regression confirmed significant associations between elevated uptake ratios and confirmed infection in every condition (OR: 22.02–245.53; all $p \leq 0.003$), which supports the use of the ratio-based framework as a reproducible, clinically actionable alternative to qualitative visual interpretation.

The demographic profile of the cohort (Table 1; median age 61 years, over half older than 60) is consistent with the epidemiological pattern of difficult-to-diagnose musculoskeletal and systemic infections, which strengthens the external validity of these results. By using a lesion-based analytical framework (346 lesions from 156 patients), the statistical power could be enhanced, and uninvolved sites could be used as internal controls. The unequal subgroup sizes (31 to 52 patients) reflect real-world referral patterns rather than predefined allocation, and the clinical heterogeneity spanning postsurgical and haematogenous aetiologies supports broader applicability of the ratio approach. Previous semiquantitative analyses of [^{99m}Tc]Tc-UBI 29-41 have relied on target-to-nontarget (T/NT) ratios using contralateral regions as a reference, with a reported cut-off of 2.55 ± 0.7 [24], representing a meaningful advance beyond visual interpretation. However, using a contralateral reference introduces variability, especially for midline structures or bilateral conditions where a true anatomical counterpart is not always available. In this study, the liver was chosen as a standardized internal reference as it offers a physiologically consistent comparator independent of lesion location. This allows a single threshold (ratio ≥ 1.5) to be applied across heterogeneous conditions, and validation through bootstrap-resampled ROC analysis and penalized logistic regression across 346 lesions provides a broader statistical basis for clinical use.

A central element of this design is that for every ratio obtained from a suspected infection site, a paired normal reference ratio was also calculated from the same patient—total counts from an uninvolved vertebral body divided by liver counts. This means each pathological ratio carries its own internal control, derived under the same acquisition conditions and reflecting the way the molecule distributes in that particular patient at that particular time point. By pairing target and normal ratios within the same individual, the approach controls for the natural variability in biodistribution as well as differences in injected dose and acquisition timing that exist between patients. The intra-patient coefficient of variation (CV) of liver counts confirmed this stability: it was below 5% in 92.3% of patients and below 10% in 96.1%, whereas the inter-patient CV was 41.7%—a figure that reflects biological and technical differences from one patient to the next but does not enter the paired analysis and therefore does not compromise the proposed cutoff. It is precisely this paired structure that allowed us to treat the ratio not merely as a semiquantitative index but as a diagnostic variable with calculable operating characteristics—sensitivity, specificity, and likelihood ratios—as reported throughout the results section. While this framework does not achieve the absolute quantification that PET/CT with ^{68}Ga - or ^{18}F -labeled UBI will eventually offer, it provides an objective, reproducible metric that reduces the intra- and inter-observer variability inherent in purely visual interpretation and can serve as a methodological foundation for future quantitative approaches.

The ROC analysis (Figure 2) revealed consistently high discriminatory capacity across all four conditions. These results are similar to those of previous reports using different analytical methods. Sepúlveda-Méndez et al. [25] reported 97.52% sensitivity and 95.35% specificity in fever of unknown origin using qualitative visual interpretation, whereas Saeed et al. [29] achieved 100% sensitivity and specificity in diabetic foot infections through a similar visual approach. Sathekge et al. [24] reported 100% sensitivity and 97.8% specificity with a cut-off of 2.55 ± 0.7 using T/NT ratios on SPECT/CT, although the results were from a limited sample with wide confidence intervals (sensitivity 95% CI: 63–100%). Direct comparison is limited by differences in populations, protocols, and reference standards, but two aspects of the present findings are worth noting: First, the consistent performance of the target-to-liver ratio across four distinct conditions rather than a single indication. Second, the plateau observed in all ROC curves at high sensitivity levels suggests that the ratio maintains diagnostic accuracy across a wide specificity range. This cross-condition consistency supports the idea of a unified ratio-based threshold as a generalizable criterion for [^{99m}Tc]Tc-UBI 29-41 SPECT/CT interpretation.

Kernel density analysis (Figure 3) demonstrated a clear separation in target-to-liver ratio distributions between infected and noninfected lesions. Using the liver as a physiological reference enabled consistent normalization across anatomically diverse conditions. Noninfected cases showed narrow distributions centred below 1.0, whereas infected lesions were shifted toward higher values. However, an overlap zone between 1.0 and 1.5 was identified, where tracer uptake may mimic physiological background, limiting discrimination between low-grade infection and sterile inflammation. Post hoc analysis further defined a three-tier classification with distinct clinical correlates. Ratios < 1.0 were highly predictive of the absence of infection, with only 1 of 29 patients classified as infected; this case corresponded to treated spondylodiscitis under antibiotic therapy, likely reflecting a bacterial burden below the detection threshold of [^{99m}Tc]Tc-UBI 29-41. Ratios ≥ 1.5 correctly identified 82 of 93 patients as infected, with 11 false positives attributable to non-infectious inflammatory conditions, including synovitis in painful arthroplasties, early postoperative spinal inflammation, and postoperative soft tissue inflammation. The intermediate range (1.0–1.49) comprised 34 patients and represented a zone of diagnostic uncertainty, including 26 noninfected cases and 8 false negatives, primarily in patients receiving antibiotic therapy or with periarticular inflammatory changes. Based on these findings, a diagnostic threshold of >1.5 was selected, prioritizing specificity ($>95\%$ across subgroups) over sensitivity to minimize false-positive diagnoses. Overall, while ratios below 1.0 and above 1.5 allow reliable classification, the intermediate interval requires integration with clinical context—particularly prior antibiotic exposure and inflammatory conditions—suggesting that this three-zone framework may be more clinically informative than a binary cut-off alone.

The Fagan nomogram analysis (Figure 4) reveals that the semiquantitative approach effectively shifts the clinical probability in both directions. Positive results increased the pretest probabilities from 8–13% up to post-test values ranging from 61% (infected arthroplasty) to 87% (spondylodiscitis), whereas negative results reduced the post-test probability to 2–3% in all conditions. This consistent rule-out capacity, regardless of the clinical scenario, suggests that [^{99m}Tc]Tc-UBI 29-41 SPECT/CT could function as a gatekeeping tool in the workup of suspected bacterial infections, in which a negative scan may reasonably allow clinicians to defer invasive confirmatory procedures, thereby supporting more judicious resource utilization and contributing to antibiotic stewardship efforts. The lower positive post-test probability in infected arthroplasty likely reflects the complexity of periprosthetic evaluation. Synovial membrane inflammation, particularly in the knee, generates diffuse uptake that can blur the distinction between septic and aseptic

processes in adjacent bones. This is consistent with the broader ratio dispersion that we observed in this subgroup (Figure 3). Conditions involving vertebral bodies, soft tissues, or pulmonary foci lack such confounding inflammatory structures, which facilitate clearer lesion delineation. Regardless of anatomical complexity, the integration of SPECT with CT remains essential, as functional images alone cannot reliably separate uptake arising from bone, synovium, or adjacent soft tissues.

The penalized logistic regression (Table 3) complements the threshold-dependent metrics by quantifying the ratio–infection association as a continuous variable. The significant odds ratios across all four conditions (all $p \leq 0.003$) confirm that this association holds true regardless of the clinical scenario. Compared with spondylodiscitis, the lower OR in infected arthroplasty (22.02) (245.53) fits the pattern observed in the ROC and Fagan analyses, where periprosthetic evaluation yielded more conservative results. The wide confidence intervals, particularly for spondylodiscitis and osteomyelitis, reflect the limited subgroup sizes and should be interpreted cautiously. Even so, the consistent directionality and statistical significance across all conditions support the validity of the ratio-based approach. Firth’s penalized method was used to mitigate the small-sample bias that affects standard logistic regression in datasets with high discriminatory capacity. These findings should be interpreted within the broader context of the current diagnostic landscape for musculoskeletal and systemic infections. [^{18}F]FDG PET/CT and radiolabelled leukocyte scintigraphy are well established in international guidelines and remain the reference functional imaging modalities for infection evaluation [3,6]. The present results do not position [$^{99\text{m}}\text{Tc}$]Tc-UBI 29-41 as a replacement for these techniques but rather suggest that it may serve as a complementary tool within the diagnostic algorithm, particularly in clinical settings where PET systems are not available. Many nuclear medicine departments worldwide operate without access to PET/CT, and a $^{99\text{m}}\text{Tc}$ -labelled molecule that can be produced using a conventional generator and imaged on standard SPECT/CT equipment represents a practical advantage for these centres. Although [$^{99\text{m}}\text{Tc}$]Tc-UBI 29-41 binds directly to bacterial and fungal membranes, its biodistribution is also influenced by the inflammatory milieu, as occurs with [^{18}F]FDG and radiolabelled leukocytes. What distinguishes the present approach is not a claim of absolute pathogen specificity but rather the integration of a direct pathogen-targeting mechanism with a semiquantitative ratio framework that strengthens diagnostic confidence, particularly in ruling out infection. As demonstrated across all four conditions, the high negative predictive value ($\geq 97.8\%$) and the consistent reduction in post-test probability to 2–3% after a negative scan represent the principal clinical contribution of this method: the capacity to reliably exclude active infection in complex diagnostic scenarios, potentially sparing patients from unnecessary invasive procedures and supporting timely, targeted antimicrobial treatment decisions. The proposed cut-off ratio of ≥ 1.5 , validated across heterogeneous conditions, provides a standardized threshold that could be readily integrated into routine clinical practice. However, each laboratory should ideally establish its own reference values, considering the distribution characteristics of the molecule over time and local acquisition conditions.

Several strengths of this study deserve mention. It is the largest prospective cohort evaluating [$^{99\text{m}}\text{Tc}$]Tc-UBI 29-41 SPECT/CT with a standardized semiquantitative method and the first study to define a specific target-to-liver ratio threshold for clinical decision-making. Although previous studies have reported similarly high sensitivity and specificity using qualitative or semiquantitative approaches, many of these analyses are limited by a lack of standardization and wide confidence intervals. In contrast, the present study provides a more robust and generalizable framework by applying a uniform ratio across multiple clinical conditions. The use of internal controls from uninvolved vertebral bodies addresses the ethical constraint of excluding healthy subjects while providing patient-specific refer-

ence values. A uniform analytical framework was applied across four clinically distinct conditions, and when combined with an integrative analytical framework, it moves beyond traditional diagnostic accuracy reporting by simultaneously addressing discrimination, clinical utility and the distributional behaviour of uptake ratios to go beyond conventional sensitivity-specificity reporting. However, future analyses using hierarchical models may further refine these estimates. A distinctive clinical advantage of [^{99m}Tc]Tc-UBI 29-41, supported by our findings, is its ability to localize infectious foci independently of the host immune response. Although radiolabelled leukocyte scintigraphy remains the reference standard, it requires specialized infrastructure and an adequate circulating white blood cell count for ex vivo labelling. In contrast, the UBI 29-41 peptide directly targets bacterial membranes. This property positions our semiquantitative framework as a valuable diagnostic alternative in leukopenic or febrile neutropenic patients, in whom cell migration-based imaging is often technically unfeasible or yields suboptimal results.

This study also has several limitations. The wide confidence intervals for sensitivity and odds ratios in some subgroups are a consequence of the limited number of confirmed positive cases and should be interpreted with caution. The composite reference standard, while clinically necessary, may introduce some misclassification bias. The single-centre design could limit generalizability, as institutional differences in acquisition protocols and equipment may influence ratio measurements. Therefore, it was not possible to perform a head-to-head comparison with [^{18}F] FDG or radiolabelled leukocytes, which limits the ability to position this approach within the existing diagnostic hierarchy. Additionally, although clustering at the patient level accounted for the lesion-based analytical framework, it may introduce residual dependence and potentially inflate precision estimates.

Multicentre validation of the proposed cut-off (≥ 1.5) is needed to test its reproducibility across different settings and populations. Prospective head-to-head comparisons with [^{18}F] FDG PET/CT and radiolabelled leukocyte scintigraphy would help clarify the comparative and complementary role of each modality. In particular, future studies using ^{68}Ga - or ^{18}F -labeled UBI 29-41 PET/CT would enable true quantitative analysis with potentially improved reproducibility across centers, representing a natural evolution of the semiquantitative diagnostic framework proposed in this work. Future work could explore serial ratio measurements as objective biomarkers for therapeutic response, particularly for guiding decisions on when to discontinue antibiotic therapy in complicated patients, where the current lack of reliable imaging criteria often leads to prolonged empirical treatment. A ratio-based approach that objectively reflects changes in bacterial burden over time could provide clinicians with a measurable endpoint to support discontinuation decisions. From a clinical standpoint, the high negative predictive value observed across all conditions suggests that this approach may be particularly useful as a rule-out test in complex diagnostic scenarios, potentially reducing the need for invasive procedures such as biopsies or surgical exploration. Finally, the application of machine learning algorithms trained on ratio data and clinical variables may help refine the diagnostic threshold and improve classification within the 1.0–1.5 overlap zone, where infection and sterile inflammation remain difficult to separate.

5. Conclusions

[^{99m}Tc]Tc-UBI 29-41 SPECT/CT with semiquantitative target-to-liver ratio analysis shows good diagnostic accuracy for identifying difficult-to-diagnose bacterial infections across infected arthroplasty, spondylodiscitis, osteomyelitis, and fever of unknown origin. A cut-off ratio ≥ 1.5 supports a potentially reproducible threshold that requires multicentre validation. These findings position [^{99m}Tc]-UBI 29-41 as a promising bacteria-specific imaging agent that could complement existing nuclear medicine techniques in the diag-

nostic algorithm for complex infectious processes. Multicentre prospective validation is warranted to confirm these results and refine the proposed diagnostic thresholds.

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Informed Consent Statement: The requirement for informed consent was waived due to the nature of the study, which involved the analysis of previously acquired imaging data without direct patient interaction. All data were fully anonymized prior analysis.

Data Availability Statement: The dataset analyzed during the current study will be made available from the corresponding author upon reasonable request and with approval from the ethics committee.

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