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Thermonuclease test accuracy is preserved in methicillin-resistant *Staphylococcus aureus* isolates

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Abstract

Introduction. The *nuc* gene encodes a thermonuclease which is present in *Staphylococcus aureus* but not in coagulase-negative staphylococci (CoNS) and is the target of the rapid phenotypic thermonuclease test. The effect of *nuc* gene variation in methicillin-resistant *S. aureus* (MRSA) on the performance of PCR testing has been noted, although there are no reports about the effect of MRSA on the activity of the thermonuclease enzyme.

Aim. Our goals were to examine the sensitivity and specificity of the thermonuclease test used to distinguish *S. aureus* from CoNS cultured from blood. In addition, we aimed to assess differences in the sensitivity, specificity and accuracy of the thermonuclease test between methicillin-sensitive *S. aureus* (MSSA) and MRSA isolates.

Methodology. We performed a retrospective analysis of 1404 isolates. Each isolate from a positive blood culture was identified as a Gram-positive coccus by microscopy then analysed with the thermonuclease test (Southern Group Laboratory) prior to confirmatory identification using VITEK microbial identification platforms (bioMérieux) and cefoxitin disc diffusion testing.

Results. Of 1331 samples included in the final analysis, 189 were thermonuclease-positive, of which 176 were identified as *S. aureus*. Of the 1142 thermonuclease-negative samples, 13 were finally identified as *S. aureus*, giving a sensitivity of 93.1% (95% confidence interval [CI] 88.5–96.3) and specificity of 98.9% (95% CI 98.1–99.4). Of the nine proven MRSA samples, eight were thermonuclease-positive, giving a sensitivity of 88.9% (95% CI 51.8–99.7). Thermonuclease test accuracy for MSSA and MRSA isolates was 98.1% (95% CI 97.2–98.8) versus 98.8% (95% CI 98.0–99.3), respectively.

Conclusions. In the era of increasing use of molecular-based microbiology assays, the thermonuclease test remains a simple, inexpensive and robust test for the presumptive identification of *S. aureus* cultured from blood, irrespective of methicillin sensitivity.

INTRODUCTION

Staphylococcus aureus can cause devastating infections and delays in disease diagnosis and management deleteriously affect patient outcomes [1]. Thermostable deoxyribonuclease (TNase), coded for by the *nuc* gene, is a specific heat-stable DNase that degrades DNA. The thermonuclease, or TNase, test detects the presence of heat-stable DNase. The test organism is first heated to destroy heat-labile thermonucleases and is then inoculated on medium containing DNA and toluidine blue. Heat-stable DNases then cleave DNA, resulting in the toluidine blue undergoing a macroscopic chromogenic change, indicating a positive result. The TNase test is a rapid (<2 h) test used to presumptively distinguish *S. aureus* present in blood cultures from coagulase-negative staphylococci (CoNS), which are negative by the TNase test. Other thermonuclease positive *Staphylococcus* strains include *S. intermedius*, subspecies of *S. schleiferi*, some strains of *S. hyicus* and *S. pseudintermedius* [2]. The TNase test is a more rapid, more sensitive and similarly

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Abbreviations: CI, confidence interval; CoNS, coagulase-negative staphylococci; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; SMI, UK Standards for Microbiology Investigations; TNase, thermostable deoxyribonuclease.

Table 1. Distribution of isolates obtained from 1331 consecutive positive blood cultures (with adequate blood volumes) over almost 4 years (March 2014 to January 2018)

TNase, thermonuclease; MSSA, methicillin-sensitive *S. aureus*; MRSA, methicillin-resistant *S. aureus*; CoNS, coagulase-negative *Staphylococcus*.

		Isolate	*n*	TNase test positive [*n* (%)]
S. aureus		MSSA	180	168 (93.3)
		MRSA	9	8 (88.9)
		Total	**189**	**176 (93.1)**
Non-*S. aureus*	**CoNS**	*Staphylococcus lugdenesis*	3	0
		Staphylococcus epidermidis	422	1 (0.2)
		Staphylococcus hominis	174	0
		Staphylococcus capitis	131	2 (1.5)
		Staphylococcus saprophyticus	7	0
		Staphylococcus warneri	19	1 (5.3)
		Staphylococcus cohnii	9	0
		Staphylococcus haemolyticus	38	0
		Mixed growth (>1 CoNS)	178	4 (2.2)
		Undifferentiated CoNS	69	3 (4.3)
	Other	*Micrococcus* species	54	0
		Undifferentiated Gram-positive cocci	26	1 (3.8)
		Non-Gram-positive cocci	11	1 (9.1)
		No growth	1	0
		Total	**1142**	**13 (1.1)**

priced alternative to the Direct Tube Coagulase test, which takes up to 4 h. TNase tests have a reported sensitivity of 96.7% and specificity of 100% [3]. However, both of these methods involve multiple steps and are labour-intensive.

The development of molecular methods, particularly real-time PCR-based tests, offers rapid identification of *S. aureus* directly from patient samples, with a sensitivity and specificity of over 95% [4]. The *nuc* gene is often the specific target of PCR-based methods for the identification of *S. aureus* [5]. However, there have been reports that variations in the *S. aureus*-specific *nuc* gene can potentially lead to misidentification of methicillin-resistant *S. aureus* (MRSA) via false-negative PCR results [6, 7]. It has therefore been suggested that *nuc*-specific PCR should not be the only molecular method for diagnosing *S. aureus* infection as it could misidentify MRSA as CoNS.

Whilst the effect of *nuc* gene variation in MRSA on the performance of molecular diagnostics and DNase testing has been noted, there have been no reports about whether MRSA isolates exhibit altered thermonuclease enzyme activity and affect the accuracy of the phenotypic TNase test.

We examined the sensitivity and specificity of the TNase test performed as part of a clinical microbiology laboratory's routine evaluation of positive blood cultures, with particular assessment of the effect of MRSA on test performance.

METHODS

We conducted a retrospective audit of the performance of consecutive TNase tests undertaken between March 2014 and January 2018 in a UK clinical microbiology laboratory providing an infection service to two urban hospitals and multiple community healthcare facilities (this study did not involve the direct use of patient samples). This laboratory processes 250 000–300 000 samples per year, including 12 000–13 000 blood cultures. Every blood culture bottle (BacT/ALERT 3D Microbial Identification System; bioMérieux) that flagged positive and showed Gram-positive cocci in clusters on Gram-staining underwent a TNase test according to the manufacturer's instructions (Thermonuclease Agar; Southern Group Laboratory). For each sample, 1 ml of positive blood culture fluid was centrifuged at 2800 ***g*** for 2 min and then incubated at 112 °C for 20 min. After 10 s of pulsed centrifugation, 100 µl of supernatant was placed in a 5 mm well cut into the TNase media and incubated at 37 °C for 2 h. Negative (*S. epidermidis* ATCC 12228) and positive (*S. aureus* NCTC 6571) controls were used, and the presence of a zone of

Table 2. Summary of the TNase test performance in the context of all *S. aureus* isolates, methicillin sensitivity and low blood volumes

TNase, thermonuclease; MSSA, methicillin-sensitive *S. aureus*; MRSA, methicillin-resistant *S. aureus*; CoNS, coagulase-negative *Staphylococcus*; CI, confidence interval; NA, not applicable.

	TNase test result	*S. aureus* (*n*)	Non-*S. aureus* (*n*)	Sensitivity [% (95% CI)]	Specificity [% (95% CI)]	Accuracy [% (95% CI)]
All *S. aureus*	Positive	176	13	93.1 (88.5–96.3)	98.9 (98.1–99.4)	98.1 (97.2–98.7)
	Negative	13	1129			
MSSA	Positive	168	13	93.3 (88.6–96.5)	NA	98.1 (97.2–98.8)
	Negative	12	1129			
MRSA	Positive	8	13	88.9 (51.8–99.7)	NA	98.8 (98.0–99.3)
	Negative	1	1129			
Low blood volume	Positive	6	3	54.6 (23.4–83.3)	90.0 (73.5–97.9)	80.5 (65.1–91.2)
	Negative	5	27			

clearing around the wells was recorded at 30 min, 1 h and 2 h. Results were recorded as positive, negative or equivocal, along with information on the nature of the sample (e.g. blood or fluid) and the volume of the blood in the initial samples (e.g. normal, low or paediatric bottle). Workup of samples followed standard operating protocols [8]. Final bacterial identification and sensitivities were established using the VITEK MS and VITEK 2 platforms, respectively (bioMérieux). Isolates identified as MRSA by VITEK MS were confirmed by cefoxitin disc diffusion testing. We assessed only adult blood culture samples, excluding all paediatric bottles and any sterile site fluids. TNase test performance was assessed by calculating the sensitivity, specificity and accuracy of the test with 95% confidence intervals (CIs) [9]. The difference in these parameters between methicillin-sensitive *S. aureus* (MSSA) and MRSA isolates was calculated using the $N-1$ chi-squared test and a P value <0.05 was considered statistically significant.

RESULTS

Over a 4-year period, 1404 consecutive TNase results were identified. A total of 32 samples (2.3%) were excluded (16 tests failed or had incompletely recorded information, nine were non-blood fluids and seven were paediatric blood cultures) and therefore 1372 samples were included in the final analysis.

Of the 1331 blood culture results with adequate blood volumes (≥10 ml), 189 were TNase-positive. Of these, 176 (93.1%) were subsequently confirmed as *S. aureus*. Of the 1142 TNase-negative results, 13 (1.1%) were identified as being *S. aureus*. A wide range of coagulase-negative staphylococci were identified, dominated by *S. epidermidis* (Table 1).

The TNase test sensitivity was 93.1% (95% CI 88.5–96.3) and specificity was 98.9% (95% CI 98.1–99.4) for all *S. aureus* isolates, irrespective of methicillin sensitivity (Table 2).

Of the 189 *S. aureus* isolates, nine were MRSA (4.8%). Among these, eight isolates were TNase-positive. In the context of MRSA, the sensitivity of the TNase test was 88.9% (95% CI 51.8–99.7). There was no significant difference in the sensitivity of the TNase test between MSSA and MRSA isolates (93.1 versus 88.9%, respectively; P=0.61). This was also the case for TNase test accuracy (98.1 versus 98.8%, respectively; P=1.0).

In addition, there were 41 low blood volume (<10 ml) blood culture samples, of which nine were TNase-positive (six *S. aureus* isolates) and 32 TNase-negative (five *S. aureus* isolates). For these low blood volume samples, the TNase test had a sensitivity of 54.6% (95% CI 23.4–83.3) and a specificity of 90.0% (95% CI 73.5–97.9) (Table 2).

There were significant differences in the sensitivity, specificity and accuracy of the TNase test between adequately filled and low blood volume samples (P<0.0001).

DISCUSSION

The rapid identification of pathogenic *S. aureus* over likely contaminant CoNS is important as it allows for the speedy initiation of targeted therapy. Our data are comparable with historical studies and show that when adequate volumes of blood are used, the TNase test remains a reliable method for the presumptive discrimination between *S. aureus* and CoNS in blood cultures [10, 11]. In this context, the overall sensitivity and specificity of the TNase test is comparable to commercially available molecular identification platforms but has the advantages of being cheap, rapid and can be performed without the need for specialist equipment. Furthermore, we found that an inadequate volume of blood collected in a blood culture bottle significantly reduces the sensitivity, specificity and accuracy of the TNase test, consistent with previous studies [12]. In contrast, the sensitivity and accuracy of the TNase test were not significantly different between MSSA and MRSA isolates. This is the first report of preserved TNase functionality in MRSA and our findings are important in view of recently raised concerns about molecular tests using the *nuc* gene as the sole target

to identify *S. aureus*. We conclude that while evidence exists to suggest that false-negative PCR results can occur with MRSA isolates, the TNase test appears robust in the presumptive identification of *S. aureus*, irrespective of methicillin sensitivity.

The TNase test described above was developed and validated by this clinical microbiology laboratory in accordance with the manufacturer's protocol over 20 years ago, before the publication of the UK Standards for Microbiology Investigations (SMI) protocol [2]. This local method has been accredited by the UK Accreditation Service (UKAS), although there are some differences between this and the SMI protocol including the centrifugation (2800 ***g*** for 2 min versus 1000 ***g*** for 10 min) and incubation (112 °C for 20 min versus 100 °C for 15 min) conditions.

Finally, while the nomenclature of the TNase genes in *S. aureus* is typically limited to *nuc*, a second thermonuclease, Nuc2, has recently been characterized following its prediction based on whole genome sequence data [13]. The two thermonucleases in *S. aureus*, encoded by *nuc1* and *nuc2*, appear to make varying contributions to the overall thermonuclease activity in *S. aureus* and have different functionalities and stabilities. It is unknown what effect, if any, this may have on the TNase test described above and we suggest this would be an area for future research.

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Author contributions
M.O., N.W. and J.S. conceived the study; M.O., B.C. and I.M. collected the data; all authors analysed the data and made significant contributions to the writing and editing of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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