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Research article

Comparison of different methods in the identification of Staphylococcusaureus in cattle

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Abstract

This study aimed to compare the efficiency of conventional microbiological , Miniaturize kit GP24 and molecular technique by using PCR of nuc gene in identification of S. aureus. Also to evaluate efficiency of the tube coagulase and DNase activity tests. 104 of suspected S. aureus were isolated from different cattle samples, (68.3%) and (75%) were positive for coagulase and DNase tests, respectively. The sensitivity and specificity of coagulase and DNase were 92.5%, 75.7% and 79.1% , 32.4% respectively. The identification rate of S. aureus by GP24 kit and PCR detection using nuc gene were 68.26% and 64.42% respectively. Out of 104(31.7%) of isolates were identified as coagulase negative staphylococci by using GP24 kit. Antimicrobials susceptibility assays of 67 S. aureus isolates revealed that, the highest resistance rates were against penicillin (97%) fallowed by tetracycline (25.4%). While all isolates were found to be highly sensitive toward Imipenem, vancomycin and chloramphenicol (100%) for each. In conclusion the coagulase and conventional microbiological techniques have more reliable results in comparison with DNase test for identification of S. aureus. On other hand, the results of GP24 kit and coagulase tube test were similar in sensitivity and specificity.

Keywords: S. aureus, GP24 kit, coagulase, DNase, nuc gene.

Introduction

Staphylococcus aureus belongs to the family Staphylococcaceae which are spherical gram positive bacteria , approximately of 0.5 – 1.5 µm in diameter, which form irregular clusters resembling bunches of grapes. aerobic, facultative anaerobic, non-spore forming and non-motile (1).S. aureus is an important etiologic agent producing infections in animals (2) and cause a wide range of infections including dermatitis, pneumonia, septicemia, osteomyelitis and meningitis as well as mastitis in cattle (3).

Routine identification and enumeration of S. aureus isolates are usually carried out by conventional

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methods based on the use of selective media such as Baird-Parker and mannitol salt agar (MSA), followed by identification of suspicious colonies by biochemical reactions which include catalase positive and oxidase negative(4, 5) and coagulase positive, which is an enzyme secreted by S. aureus causing the clotting of plasma in the host(6, 7). Coagulase production is an important phenotypic determinant of S. aureus which is associated with virulence as it resists phagocytosis and helps bacteria in virulence (8). Tube coagulase test detects secreted extracellular free coagulase that reacts with a substance in plasma called "Coagulase-Reacting Factor" (CRF) to form a complex, which in turn reacts with fibrinogen to form fibrin (the clot) (9).

Seven species of coagulase-positive staphylococci (CoPS) have been identified including Staphylococcus aureus, S. intermedius, S. schleiferi subsp. coagulans, S. hyicus, S. lutrae, S. delphini, and S. pseudintermedius . In addition to S. aureus, the other CoPS species can cause severe infections compared with those caused by coagulase-negative staphylococci (CoNS) (10).

S. aureus strains produce an extracellular thermostable nuclease protein with a molecular mass of 17,000 Da. encoded by nuc gene. It is an endonuclease, degrading both DNA and RNA(86R), this gene is a specific marker gene that can be used for distinguishing S. aureus from other staphylococcus spp.(11,12).

This study aimed to compare the efficiency of conventional microbiological, Miniaturize kit GP24 and molecular technique (PCR of nuc gene) in identification of S. aureus. Also to evaluate the efficiency of the tube coagulase and DNase activity tests.

Materials and Methods

Ethical approval

The study was approved by the College of Veterinary Medicine; University of Basrah, Basrah, Iraq

Samples collection: A total of 212 samples from cattle including ; 42 mastitis milk samples detected by using California mastitis test (CMT) according to (13), the study was conducting during the period from January to June in Basrah (2018) as shown in table 1.

Table (1): Number and types of collected samples

Samples	Number
Mastitic milk (detected by CMT)	42
Raw milk from local market	70
Nasal swabs	46
Udder surface swabs	32
Coat Swabs	22
Total	212

Conventional microbiological technique:

All samples were subjected to colony morphology characteristics on selective media(MSA), Gram ' s staining, conventional biochemical tests (catalase, oxidase, DNase and coagulase) according to (14).

Miniaturize kit GP24:

The suspected isolates were submitted to GP24 (Slovak, slovakia) a ready-to-use test consisting of 24 biochemical tests to which an homogenous bacterial suspension (100µl) at 3McFarland turbidity is added. Urea (URE) and Arginine (ARG) wells (H1 and H2 wells) were covered with 2 – 3 drops of paraffin oil. The plate was incubated at

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37ºC for 24 h. Result of identification was obtained by identification table and identification online software (DIAGNOSTICS s. r. o.)

Molecular detection:

Genomic DNA of suspected S. aureus isolates was extracted by DNA kit (Geneaid, USA) and according to manufacturer's protocol. S. aureus isolates were confirmed by PCR detection using nuc gene(423bp) primer nuc

F: 5´- GCT TGC TAT GAT TGT GGT
AGC C 3' and

R: 5´- TCT CTA GCA AGT CCC TTT
TCC A 3' according to (15).

Determination of the antimicrobial susceptibility of isolates:

The confirmed S. aureus isolates were subjected to antimicrobial susceptibility test. Twelve antimicrobials from (Bioanalyse/ Turkey) were used to detect the isolates susceptibilities using disk diffusion method according to (16) including, Cefoxitin (30 µg), Ceftriaxone (30 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Erythromycin (15 µg), Gentamycin (10 µg), Imipenem (10µg), Oxacillin (1 µg), Penicillin (10 µg), Tetracycillin(30 µg), Trimethoprin(5 µg) and Vancomycin (30 µg). Cefoxitin was used as a surrogate for study of methicillin resistance.

Statistical analyses

The data were statistically using analyzed the software package IBM SPSS 22. Next a 2 × 2 contingency table was used for diagnostic specificity and sensitivity . Diagnostic sensitivities and specificities were calculated as follows (17):

Sensitivity = TP/ (TP+FN)

Specificity = TN/ (TN+FP)

*TP :true positive, FP: false positive, TN: true negative, FN: false negative **Results**

Out of 212 tested samples 104 (49.1%) isolates were considered as suspected S. aureus by using conventional microbiological techniques. Out of 104 suspected isolates, 71(68.3%) and 78(75%) were positive for coagulase and DNase tests, respectively, as shown in table (2) and figure (1). The sensitivity and specificity of DNase were 79.1% and 32.4% respectively. Whereas, for coagulase were 92.5% and 75.7% respectively. However the sensitivity and specificity of coagulase and DNase tests were calculated in comparison with detection of nuc gene (table 2).

Table (2) Number of isolates which identified by using different diagnostic methods

Sample tested	No. of Isolates identified by *	Dnase test (%)	Coagulase (%)	nuc gene (%)
212	104	(75)	(68.3)	(64)

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* Culturing on MSA, Gram stained smear and biochemical tests (Oxidase and catalase tests). Sensitivity and specificity of coagulase test= 92.5%, 75.7%.
Sensitivity and specificity of Dnase test=79.1%, 32.4%.

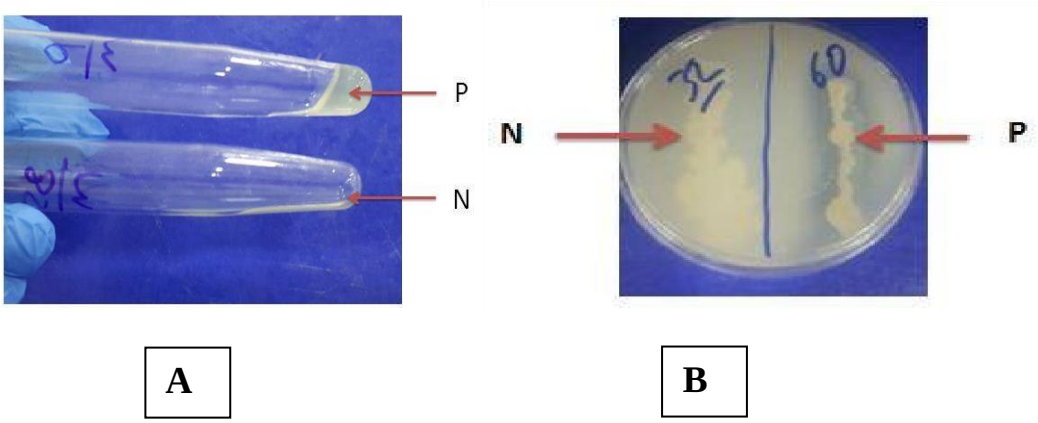

Figure (1) A: Tube Coagulase Test, P: coagulase positive (clot), N: coagulase negative (not clot);B:DNase test, P: positive result and N: negative result

All suspected isolates (104) were subjected to identification using GP24 kit figure (2) and PCR detection using nuc gene figure (3). The identification rate of S. aureus was 68.26% and 64.42% respectively table (3). By using molecular detection, the results showed that, the high percentage of S. aureus isolates were from udder surface samples (93.33%), followed by subclinical mastitis (64.28%), whereas the lowest percentage was observed from the coat swabs (55.55%). The sensitivity and specificity of GP24 kit were 92.5% and 75.7% respectively. The sensitivity and specificity of GP24 kit to identify S. aureus was calculated in comparison with detection of nuc gene

Table(3): Comparison between conventional microbiological techniques, GP24 kit and moleculartechnique in identification of S. aureus from different samples sources.

Samples	No. of S. aureus using		
	conventional microbiological technique	GP24 kit	nuc gene
Subclinical mastitis	28	(64.28%)	(64.28%)
Raw milk from local markets	37	(72.97%)	(56.75%)
Nasal swabs	15	(66.66%)	(60.00%)
Udder surface swabs	15	(73.33%)	(93.33%)
Coat swabs	9	(55.55%)	(55.55%)
Total	104	(68.26%)	(64.42%)

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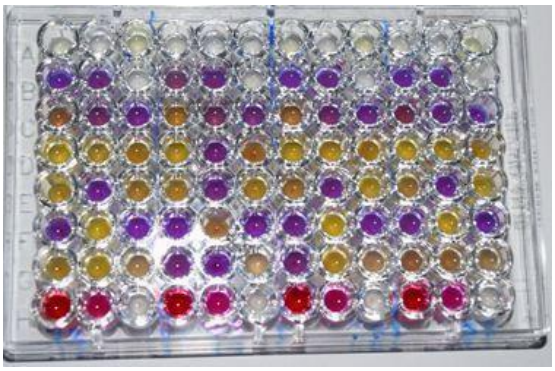
Sensitivity and specificity of GP24 kit = 92.5%, 75.7%.

*****The ratios were calculated by dividing the number of cell on number of conventional microbiological technique cell.



Taxa	Identification (%)	Differentiation (T)
Staphylococcus aureus ssp. aureus	excellent 100.00	excellent 1.00

	CAT	URE	MLT	SOR	LAC	FRU	ARA	RAF	bGA
	+	+	+	-	+	+	-	-	+
	OK	OK	OK	OK	OK	OK	OK	OK	OK
COA	NOV	ARG	MAN	TRE	CEL	MNS	RIB	MLZ	GLR	PHS
+	0	+	+	+	-	+	-	-	-	0
OK		OK	OK	OK	OK	OK	OK	OK	OK	
	bHEM	NIT	ESL	MLB	SUC	GAL	XYL	NAG	bGL	HIP
	0	+	-	-	+	+	-	+	+	0
		OK	OK	OK	OK	OK	OK	OK	OK	
		OXI	VP	PYR	LAP	BAC	YEP	AHE		
		-	0	0	0	0	0	0		
		OK								

Figure (2) A: The plate of GP24 kit B: Online identification table using GP24 kit (The plates designed for 4 isolates)

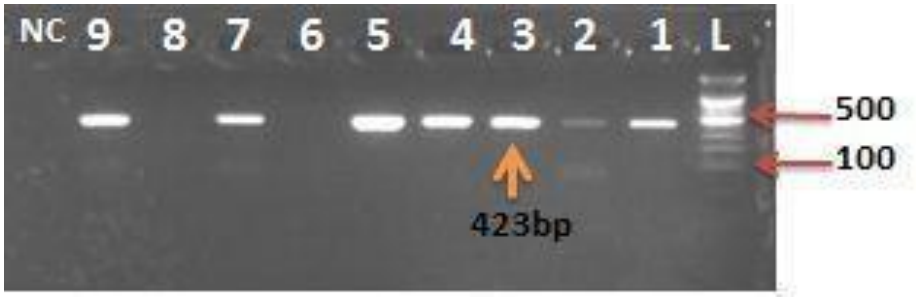


Fig (3): Electropherogram of nuc gene amplification.

The product size (423) bp, the mixture was run in1.5 % agarose gel, stained with ethidium bromide, L: Ladder.

The104 isolates (68.26%) were identifiedas S. aureus and the rest (31.7%) were identified as the other Staphylococcus spp by using miniaturize GP24, (30.3%), (27.3%), (27.3%), (12.1%) and (3.0%) of isolates were identified as S. chromogenes, S. hominis, S. xylosus, S. capitis and S vitulinus respectively (table 4).

Table (4): Number and percentages of other Staphylococcal spp. identified by using GP24 kit.

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	isolates (%)	milk of market isolates (%)	isolates(%)	isolates(%)	isolates (%)	%
S. hominis	3	3	3	0	0	27.3%
S. xylosus	4	2	1	2	0	27.3%
S. chromogenes	4	3	0	2	1	30.3%
S. vitulinus	0	1	0	0	0	3.0%
S. capitis	0	0	0	2	2	12.1%
Total	33.3%	27.3%	12.1%	18.2%	9.1%	33

* The ratios were calculated by dividing the number of cell on total number cell

The results showed that, the highest resistance was against penicillin (97%) fallowed by tetracycline (25.4%), Ceftriaxone (20.9%) and Ciprofloxacin (18%). While all isolates were found to be highly sensitive toward Imipenem (100%), vancomycin (100%) and chloramphenicol (100%). Out of 67 isolates 44(65.7%) were resistant to Cefoxitin, whereas, 48(71.64%) were resistant to oxacillin, Cefoxitin were used to detect the phenotype of methicillin resistant Staphylococcus aureus (MRSA). Table (5)

Table (5): Antimicrobial susceptibility of S. aureus isolates from

Antimicrobial agents	Sensitive	Intermediate	Resistance
Ceftriaxone	(52.2%)	(26.9%)	(20.9%)
Chloramphenicol	(100%)	(0%)	(0%)
Ciprofloxacin	(71.6%)	(10.4%)	(18%)
Erythromycin	(56.8%)	(32.8%)	(10.4%)
Gentamycin	(80.6%)	(11.9%)	(7.5%)
Imipenem	(100%)	(0%)	(0%)
Penicillin	(3%)	-	(97%)
Tetracycline	(40.3%)	(34.3%)	(25.4%)
Trimethoprim	(55.2%)	(28.4%)	(16.4%)
Vancomycin	(100%)	(0%)	(0%)
Cefoxitin	(34.3%)	-	(65.7%)
Oxacillin	(22.4%)	(5.9%)	(71.64%)

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Discussion

The initial identification of S. aureus depended on colonial appearance on the conventional selective media MSA, their biochemical profile and confirmed by molecular method (PCR) (18).In this study the suspected isolates using conventional microbiological techniques were (49.1%), this result was compatible with (19) who reported that (40.81%) of samples were carried S. aureus. Whereas this result was higher than (20), who revealed that S. aureus was isolated from (37%) raw milk of cow in Basrah , and lower than (19) who detected 85.7% of suspected S. aureus by using conventional microbiological techniques.

Out of 104 isolates (68.3%) were coagulase positive, The current result is compatible with the studies of (21, 22) who reported coagulase production in percentages 78.5% and 77.8% respectively. While, higher percentage 89% was recorded by (18). In contrast, a much lower percentages were reported by the studies of(23 , 24) 28.07% and 16.67%, respectively. Sensitivity and specificity of coagulase test were evaluated by comparing with nuc gene, the results were 92.5% and 75.7% respectively. Similar results were obtained by (18) who revealed that sensitivity of tube coagulase test was more than 91 % and (25) who reporting a sensitivity of 97% but a specificity of 100%. Higher percent of coagulase positive isolates comparing to nuc gene result may be due to present other coagulase positive staphylococcus spp.

The DNase and thermostable nuclease activity (TNase) tests are being used for presumptive identification and appear to be a consistent property of S aureus (26). In present investigation 78(75%)showed DNase activity on the DNase agar, this result is in agreement with (18) who reported 75% of cultures were positive for DNase test, however, this result is lower than (21) who found DNase activity in 86.93%, and higher than (23) who reported (65.7%). On the other hand DNase test had a sensitivity of 79.1% and specificity 32.4%. congruous, in result of sensitivity was obtained by (18, 27) who reported 75% and a 96%. Concerning the specificity, the present result is consistent with (28) who reported the sensitivity and specificity of DNase as 53.13% and 41.84% respectively .

Seventy one out of 104 isolates (68.3%) were identified by Miniaturize kit GP24 as S. aureus and the rest (31.7%) of isolates were identified as coagulase negative staphylococci (CoNS). Higher occurrence rates of CoNS were observed in both mastitic and raw milk 11(33.3%) and 9(27.3%) respectively , similar result was obtained by (29 and 30)who recorded the isolation rates of CoNS from bovine subclinical mastitis ranged from 7.4% to 53.5% . CoNS are normal flora of the skin of the teat and common in subclinical mastitis (31). This result was higher in compared with the study of (32) who reported the isolation rate of CoNS from subclinical mastitis was 15.55%. The sensitivity of GP24 kit was high 92.5%, and specificity was 75.7%. On other hand this results are similar to result of coagulase test.

Concerning the molecular detection of S. aureus by pcr technique, targeting of nuc gene encodes for a specific thermostable nuclease (33). This gene were used to detect S. aureus in previous studies (34, 35. 36). In present study 67 (64.42%) of isolates were identified as S. aureus,

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This result is in line with many studies such as (37, 38) In contrast, a much lower occurrence rates of S. aureus were found by (39, 40, 41).The highest rates of S.aureus were observed in udder surface and nasal swabs (93.33% and 60%, respectively),this result explains the causes of mastitis may be due to smudge the teats with dung and mud, also the direct source of bacteria of milk.While the causes which increase this microorganism in nares may be due to warm, moist and nutrients.

The antimicrobial susceptibility test profile obtained in the present study showed that a significant percentage of S. aureus isolates were resistant to most of the commonly used antibiotics such as pencillin, Oxacillin and Cefoxitin. The cefoxitin diffusion method was used as an alternative marker for the detection of methicillin resistance (16), the resistance of Staphylococcus spp. to penicillins is a well-known phenomenon worldwide, and 10 to 70 % of S. aureus in cows may be resistant to these drugs, depending on geographical location (42). This result compatible with local study (43). However(44) found that resistant to penicillin was (76.92%) followed by gentamicin(51.28%), oxacillin (38.46 %), tetracyclin (28.21%) and erythromycin (23.08%) in Staphylococcus species from mastitic milk.

On other hands, all the S.aureus isolates were sensitive to vancomycin chloramphenicol and Imipenem (100%), the present study are in line with studies (45, 46) .Many factors may have contributed to the above level of resistance toward the tested antibacterial drugs. Three major factors determine this crisis, the increasing frequency of antimicrobial resistance phenotypes among microbes is an evolutionary response to the widespread use of antimicrobials, the resistance of bacteria may be resulted from arbitrary uses of antimicrobial and the extensive and often unnecessary use of antimicrobials by humanity provides the strong selective pressure that is driving the evolutionary response in the microbial world (47).

In conclusion, the coagulase plus conventional microbiological techniques have more reliable results in comparison with DNase test and can be used for initial identification of S. aureus. However, the DNase test plus conventional microbiological techniques may use as screening test for primary isolation of S. aureus. Concerning GP24 kit, the results were correlated with coagulase test because this kit depend on adding the result of coagulase to identify the gram positive bacteria, moreover, the sensitivity and specificity of GP24 similar to that of coagulase test . The number of S. aureus isolates which identified using GP24 kit, coagulase or DNase were more than that identified by using nuc gene, these results were due to the presence of other coagulase positive staphylococci which have phenotypes similar to S. aureus.

Conflict of interest

No conflict of interest is found.

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