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Original Research Article

Characterization of antimicrobial resistant bacterial pathogens recovered from cases of bovine mastitis with special reference to *Staphylococcus aureus*

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ABSTRACT

In the current study, a total of 20 and 78 milk samples were collected from animals showed signs of clinical and subclinical mastitis, for isolation and identification of different causative pathogens in some dairy farms of Beni-Suef Governorate, and for investigation of *in vitro* sensitivity. The recovered microorganisms were *Staphylococcus* species (*n*=79; 80.61%), *Enterococcus* spp. (*n*=28; 28.57%), CAMP negative Streptococci, *Pseudomonas aeruginosa* (*n*=7; 7.14%), *E. coli* (*n*=3; 3.06%) and *Proteus vulgaris* (*n*=1; 1.02%). Antibiogram profile for *S. aureus* showed that the most effective drug was vancomycin and the least was penicillin. Trials were done to detect biofilm production for recovered isolates of *S. aureus* (*n*=23) by the use of a phenotypic method (Congo red agar, CRA) and genotypic methods through determination of some biofilm related genes using PCR. All recovered *S. aureus* isolates were seeded on the CRA media to detect the biofilm forming ability. It has been found that all tested isolates showed a biofilm forming ability either strong (13; 56.52%) or intermediate (10; 43.48%). The detection of some biofilm associated genes (*icaA*, *icaD* and *bap* genes) using polymerase chain reaction revealed that two (10.53%) isolates out of 19 were negative for all tested genes, 16 (84.21%) isolates harbored both *ica*A and *ica*D gene, while only one (5.26%) isolate had all tested genes.

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1. Introduction

Mastitis is defined as an inflammation of the mammary gland that caused by the invasion of pathogens via the teat orifice leading to intramammary infection (IMI) resulting in local and systemic symptoms (Tremblay et al., 2014).

Mastitis manifests either as subclinical, with no visible symptoms, or clinical, with visible symptoms, varying from mild (flakes in milk, slight swelling of infected quarter) to severe (abnormal milk secretions, hot swollen quarter/udder, fever, rapid pulse, loss of appetite, depression and death) (Schroeder, 2012).

Mastitis may be caused by over 250 microorganisms (Bhuvana and Shome, 2013); therefore, the detection of such pathogens is being essential for the definitive diagnosis of mastitis. Environmental pathogens (*Escherichia coli*), *Pseudomonas aeruginosa*, different *Streptococcus* species and *Staphylococcus aureus* are predominant etiological agents of both subclinical and clinical forms of udder inflammation (Osteras, 2005; Barkema et al., 2006). Furthermore, the role of coagulase negative staphylococci (CNS) has recently increased as major causes of subclinical mastitis (Khan et al., 2003). Meanwhile, enterococci are significant causes of clinical mastitis in dairy herds and the most predominant isolated species are *E. faecium* and *E. faecalis* (Smith and Hogan, 1993, 1995).

Bovine mastitis caused by *S. aureus* remains a significant problem for milk producers worldwide (Darwish and Asfour, 2013). Previous literature proved that in bovine mastitis caused by *S. aureus*, the ability to produce biofilm (slime) is the most important reason for unusual problems with eradication of infection and recurrent infections of mammary glands (Melchior et al., 2006b). Production of slime enables adhesion of bacteria to the mammary glands epithelia. It facilitates the persistence of microorganisms in the host tissue by protecting the bacterial cells against the mechanisms of the host defense. Accordingly, it causes the evident reduction of susceptibility to antibiotics, due to altered growth rate and delayed penetration of antimicrobial agents within the biofilm structure (Melchior et al., 2006a, 2007).

The production of biofilm requires the presence of the gene cluster *icaADBC* (the intracellular adhesion locus) and strains harboring the *icaADBC* cluster are potential biofilm producers (Cramton et al., 1999). Furthermore, it has been found that the biofilm-associated protein encoded by *bap* gene instead of PIA was indispensable for the primary attachment and cells' accumulation (Cucarella et al., 2001; Lasa and Penadés, 2006).

Therefore, the aim of this study was the characterization of antimicrobial resistant *S. aureus* and other bacterial pathogens isolated from mastitic cow`s milk. Consequently, the following steps well be applied:

1- Isolation and identification of different pathogens causing clinical and subclinical mastitis in some dairy farms of Beni-Suef Governorate.
2- Evaluation of the antimicrobial susceptibility behavior of *S. aureus* using disc diffusion method.
3- Phenotypic detection of biofilm production by *S. aureus* isolates using Congo red agar method.
4- Detection of some biofilm associated genes (*icaA*, *icaD* and *bap* genes) using polymerase chain reaction.

2. Materials and methods

2.1. Animals

A total of 400 cows from 6 different farms in Beni-Suef Governorate were examined for signs of mastitis (swelling, hotness, redness and apparent milk change) while the apparently healthy animals were screened by CMT for detection of subclinical mastitis.

2.2. Milk Samples

A total of 20 and 78 milk samples were collected from animals suffered from clinical and subclinical mastitis.

2.3. Bacteriological examination

2.3.1. Cultivation of milk samples

Milk samples were collected for bacteriological examination under aseptic condition according the procedure recommended by Quinn et al. (2002).

Samples were incubated aerobically at 37^{o}C for 18-24 h, then, centrifuged at 3000 rpm for 20 min. The cream and supernatant fluid were discarded. Loopfuls from the sediment were taken and streaked onto the surface of the following media, modified Edward's medium, Baird Parker and MacConkey's

agar. The inoculated plates were incubated at 37ºC for 24-48 h.

Morphological and biochemical identification of recovered pathogens were carried out according to Colle et al. (1996) and Quinn et al. (2002)

2.3.2. Antimicrobial susceptibility test

Disc diffusion technique was used to identify the antimicrobial susceptibility of the *S. aureus* isolates and interpretation was carried according to (CLSI, 2013). The following antimicrobial discs were used ciprofloxacin (CIP 5µg), cefoxitin (FOX 30µg), doxycycline (DO 30µg), gentamicin (CN 10µg), penicillin (P 10 iu), rifampicin (RD 5µg), spectinomycin (SH 100µg) and vancomycin (VA 30µg).

2.3.3. Phenotypic detection of biofilm production on Congo red agar (Vasudevan et al., 2003; Mathur et al., 2006; Dubravka et al., 2010)

CRA plates were prepared using Tryptic Soy agar containing 0.08% Congo red (Sigma). The inoculated CRA plates were incubated at 37ºC in aerobic conditions for 24 h, followed by storage at room temperature for 48 hrs. Isolates were interpreted according to their colony phenotypes. Black colonies with dry consistency and rough surface were considered positive slime production. Black colonies with smooth, round and shiny surface as well as red colonies of dry consistency and rough surface were considered intermediate slime producers. Red colonies with smooth, round, and shiny surface were considered negative slime production.

2.3.4. Genotypic Analysis of some biofilm associated genes (*ica*A, *icaD* and *bap* genes) using PCR

A total volume of 25 µl was prepared according to Emerald Amp GT PCR master mix (Takara, Japan) Code No. RR310A kit .Temperature and time conditions of the used primers were carried out according to specific authors and Emerald Amp GT PCR master mix kit (Table 1).

Table 1. List of primers used for detection of *ica*A, *ica*D and *bap* genes

Primer	Sequence (5'-3')	Product (bp)	Reference
*ica*A	F-CCT AAC TAA CGAAAG GTA G	1315	**Ciftci et al., 2009**
	R-AAG ATA TAG CGATAA GTG C		
*ica*D	F-AAA CGT AAG AGAGGT GG	381	
	R-GGC AAT ATG ATCAAG ATA		
Bap	F-CCCTATATCGAA GGTGTAGAATTG	971	**Cucarella et al., 2001**
	R-GCTGTTGAAGTTA ATACTGTACCTGC		

3. Results and discussion

In the present work, a total of 20 and 78 milk samples were collected from animals showed clinical and subclinical mastitis. It has been revealed that the most prevalent microorganisms recovered were staphylococcal species (*n*=79; 80.61%) followed by *Enterococcus* spp. (*n*=28; 28.57%), CAMP negative sterptococci, *P. aeruginosa* (*n*=7;

7.14%), *E. coli* (*n*=3; 3.06%) and *Proteus vulgaris* (*n*=1; 1.02%).

Staphylococcal species (*n*=79; 80.61%) were characterized as coagulase negative staphylococci (*n*=45; 45.92%), *S. aureus* (*n*=23; 23.47%) and *S. intermedius* (*n*=11; 11.22%) (Table 2).

Table 2. The prevalence of isolated bacterial species recovered from clinical and subclinical mastitic milk samples

Bacterial species	Clinical mastitis (*n*=20)		Subclinical mastitis (*n*=78)		Total (*n*=98)	
	No.	%	No.	%	No.	%
CNS	7	35	38	48.72	45	45.92
S. aureus	5	25	18	23.07	23	23.47
S. intermedius	3	15	8	10.25	11	11.22
Enterococcus fecalis	1	5	14	17.95	15	15.31
Enterococcus faceium	-	0	8	10.25	8	8.16
Enterococcus durans	-	0	3	3.85	3	3.06
Enterococcus avium	-	0	2	2.56	2	2.04
Pseudomonas aeruginosa	3	15	4	5.13	7	7.14
***Sterptococcus* spp.**	1	5	5	6.40	6	6.12
E. coli	1	5	2	2.56	3	3.06
Proteus vulgaris	1	5	-	0	1	1.02
Total	22	110	102	130.70	124	126.5

No. Number of recovered isolates.

% The percentage of recovered isolates relative to examined milk samples.

Such findings were in agreement with previous literature (Ferguson et al., 2007; Cervinkova et al., 2013). Similarly, Akram et al. (2013) stated nearer results except a higher prevalence for *Escherichia coli*. Meanwhile, Belayneh et al. (2014) coincided with the current finding with a lower percentage was recorded for CNS.

In the current investigation, special attention was given to *S. aureus* as it is still one of the most significant problems for milk producers worldwide (Darwish and Asfour, 2013). Some *S. aureus* strains, causative agents of mastitis in cattle, exhibit the ability to produce a viscous extracellular polysaccharide layer (slime). The latter is nowadays considered to be a virulence factor, as it promotes bacterial adhesion onto the mammary epithelial cells and protects bacteria from phagocytosis. Some strains of such genus are believed to exist in the form of a biofilm in the udder tissue, partly explaining frequent therapeutic failures and the chronic course of infection (Dubravka et al., 2010). It causes the evident reduction of susceptibility to antibiotics, due to altered growth rate and delayed penetration of antimicrobial agents within the biofilm structure (Melchior et al., 2006a, 2007).

The biofilm formation involves two sequential steps: adhesion of cells to a surface shadowed by cell-cell adhesion, creating several layers of cells (Cramton et al., 1999). Intercellular adhesion requires the polysaccharide intercellular adhesin (PIA) or polymeric N-acetyl-glucosamine (PNAG) which encoded by the *ica* operon (*ica*ABCD) (Götz, 2002), among them *ica*A and *ica*D genes have been reported to play a major role in the biofilm formation in *S. aureus* isolated from bovine mastitis (Vasudevan et al., 2003).

Moreover, some proteins named, biofilm-associated proteins (*bap*) which encoded by *bap* gene are recognized to donate in the construction of *S. aureus* communities (Latasa et al., 2006).

Treatment with antibiotics is one of the most important components to control mastitis. Due to the indiscriminate use of antibiotics, resistance has been developed against most of the commonly used drugs. The incorrect use of antimicrobials has been implicated as the major selective force for the development of resistance (Levy, 2002).

Therefore, determination of etiological agents and their antimicrobial sensitivity prior to treatment facilitates selecting suitable and cost-effective antibiotic for proper treatment of affected animals (Charaya et al., 2014). Hence, this study targeted to evaluate the antimicrobial susceptibility behavior of the recovered *S. aureus* against the most commonly used antimicrobial agents using disc diffusion method.

It has been found that all *S. aureus* isolates were sensitive to vancomycin (100%) and the majority of isolates showed a high sensitivity to ciprofloxacin, doxycycline (73.9%), gentamicin (69.5%) and rifampicin (60.86%). On the other hand, *S. aureus* isolates were highly resistant to penicillin (82.6%), cefoxitin (47.8%), spectinomycin (21.7%), with lower resistance was expressed by doxycycline, gentamicin and rifampicin (Table 3).

Table 3. The response of isolated *S. aureus* to various chemotherapeutic agents

Antimicrobial agents	Conc. (µg)	*S. aureus* (*n*=23)					
		Sensitive		Resistant		Intermediate	
		No.	%	No.	%	No.	%
Vancomycin	30	23	100	-	0	-	0
Ciprofloxacin	5	17	73.91	1	4.35	5	21.74
Doxycycline	30	17	73.92	3	13.04	3	13.04
Gentamicin	10	16	69.57	2	8.69	5	21.74
Rifampicin	5	14	60.87	3	13.04	6	26.09
Cefoxitin	30	12	52.17	11	47.83	-	0
Penicillin	10 (iu)	4	17.39	19	82.61	-	0
Spectinomycin	100	4	17.39	5	21.74	14	60.87

No. Number of positive isolates.

% Percentage was calculated in relation to the total isolates.

It is notable that the highest incidence of resistance was recorded against penicillin followed by cefoxitin. Oppositely, the highest sensitivity was detected to vancomycin. Such results are consistent with previous literature (Li et al., 2009; Irena et al., 2011; Zhang et al., 2012; Cervinkova et al., 2013)

In the present study, a multiple drug resistance (MDR) was detected amongst 6 isolates (28.09%). Several reports described MDR against *S. aureus* (Shitandi and Sterneesjo, 2004; Shi et al., 2010; Zanette et al., 2010; Kumar et al., 2011).

Methicillin-resistant *S. aureus* (MRSA) infection has recently emerged among animals and can be spread between cows and human, posing a potential risk for both human and animal health (Juhász-Kaszanyitzky et al., 2007). MRSA strains are frequently resistant to a variety of β-lactam antimicrobial agents, with the exception of the newer cephalosporins with anti-MRSA activity (CLSI, 2013). Furthermore, the presence of MRSA does not cause a delayed treatment but may cause failure of treatment (Soo Ko et al., 2005).

Currently, cefoxitin disk diffusion test was employed for phenotypic characterization of MRSA. This test is able to foretell the presence of *mec*A gene in *S. aureus* with a high degree of sensitivity and specificity (Swenson et al., 2005; CLSI, 2013). The present work revealed that out of 23 tested isolates, 11 (47.83%) was categorized phenotypically as MRSA. Such findings come in harmony with the previous literature showing that the prevalence of MRSA among *S. aureus* isolates was as high as 52% between 2003 and 2005 in Egypt (Falagas et al., 2013) A regional study carried by Elhaig and Selim (2014) has reported MRSA amongst 52.2% of the tested *S. aureus* isolates.

In the present investigation, trials were done to detect biofilm production for 23 isolates of *S. aureus* recovered from milk of bovine mastitis by the use of phenotypic methods including CRA and genotypic methods by determination of some biofilm related genes using PCR.

The detection of slime production using phenotypic methods is qualitatively, depending on morphology of colonies produced on CRA. Variation in previous literature was apparent concerning the interpretation of CRA test.

In such concern, both bright black colonies (Citak et al., 2003) and black colonies (Oliveira et al., 2006; Jain and Agarwal, 2009) were considered positive results. However, Cucarella et al. (2004) described the dry crystalline surface (rough colony morphology) as a positive result, disregarding the color (black or pink).

Such difference may possibly be due to the fact that the test was not originally designed for investigating *S. aureus* isolates (Freeman et al., 1989).

In the current investigation, the interpretation of results was carried out according to Dubravka et al. (2010) where isolates that formed black/rough colonies were recorded as strong slime producing. The smooth black or dry red colonies were considered as indeterminate producers unlike those forming red/smooth colonies are non-slime producers.

All tested isolates showed biofilm forming ability either strong or intermediate. Thirteen (56.52%) of them were strong biofilm producer which appeared as black dry colonies on CRA media. Ten (43.48%) isolates were intermediate biofilm-producer appeared as red and dry colonies.

The recorded prevalence agreed with those obtained by Vasudevan et al. (2003). Lower incidences were previously reported (Ciftci et al., *2*009; Dhanawade et al., 2010; Fabres-Klein et al., 2015).

The detection of some biofilm-related genes by means of PCR illustrated in figures 1, 2, & 3 revealed only two (10.53%) isolates out of 19 were negative for all the tested genes, 16 (84.21%) isolates harbored both *ica*A and *ica*D gene while only one (5.26%) isolate harbored all the tested genes (Table 4).

Table 4. The occurrence of biofilm-related genes among tested *S. aureus* isolates

Tested *S. aureus* isolates	Tested genes		
	bap	***ica*A**	***ica*D**
1	-	+	+
2	-	+	+
3	-	+	+
4	-	+	+
5	-	+	+
6	-	+	+
7	-	+	+
8	+	+	+
9	-	+	+
10	-	+	+
11	-	-	-
12	-	+	+
13	-	+	+
14	-	-	-
15	-	+	+
16	-	+	+
17	-	+	+
18	-	+	+
19	-	+	+

(+) the presence of the tested gene
(-) the absence of the tested gene

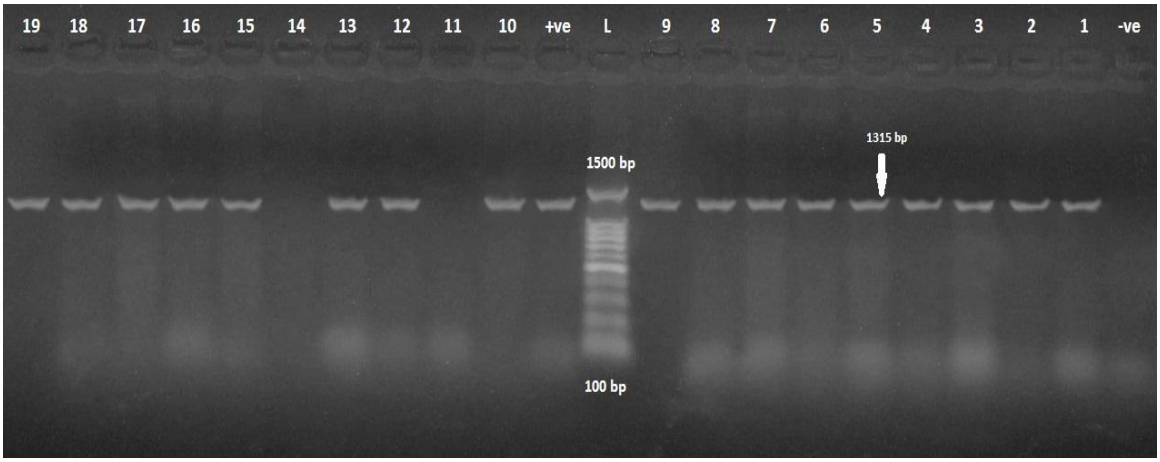

Figure 1. Agarose gel electrophoresis showing the amplification of *ica*A at amplicon of 1315 bp. Lane (1-9, 10, 12, 13 and 15-19: showed positive samples. Lane (11, 14): showed negative samples. L: Molecular size ladder. (+ve): Control positive, (-ve): Control negative

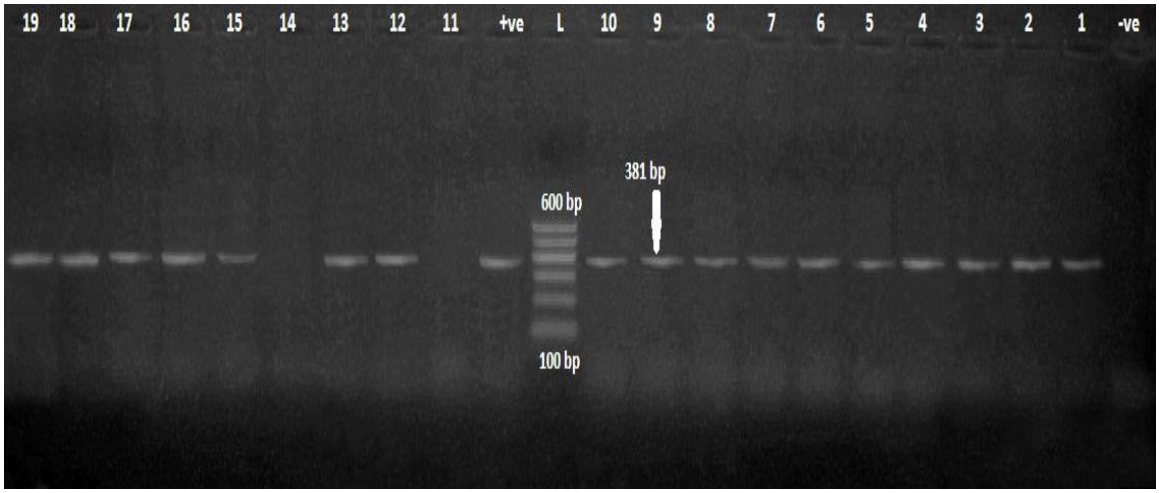

Figure 2. Agarose gel electrophoresis showing the amplification of *ica*D at amplicon of 381 bp. Lane (1-10, 12, 13 and 15-19): showed positive samples. Lane (11, 14): showed negative samples. L: Molecular size ladder . (+ve): Control positive. (-ve): Control negative.

It is notable that both *ica*A and *ica*D gene were present in 17 (89.47%). Such high prevalence is closer to that of Melo et al. (2013) who detected *ica*A and *ica*D genes in 95.7% of isolates and Castelani et al. (2015) who detected *ica*A and *ica*D genes in 98% and 100% of isolates, respectively. The obtained results go in parallel to what reviewed by Darwish and Asfour (2013) showing a high prevalence of *Staphylococcus* biofilm producers among bovine mastitis in Egypt. A lower prevalence was recorded by Ciftci et al. (2009) and Dhanawade et al. (2010).

It was notable also that the lowest prevalence among tested genes was for *bap* gene (only one isolate; 5.26%) agreeing with several literature, and *bap* is a newly identified gene and has only been found in a small proportion of *S. aureus* strains (Cucarella et al., 2004; Vautor et al., 2008; Darwish and Asfour, 2013; Goyal et al., 2014).

Remarkably the results of biofilm related genes were not well correlated with the biofilm production using CRA method. Comparing with molecular analysis, results of the phenotypic tests for biofilm formation revealed that the sensitivity and specificity of Congo red agar test were 88.9% and 100%, respectively (Melo et al., 2013). Similarly, Mathur et al. (2006) found that CRA is not recommended for the detection of biofilm formation by staphylococci alone.

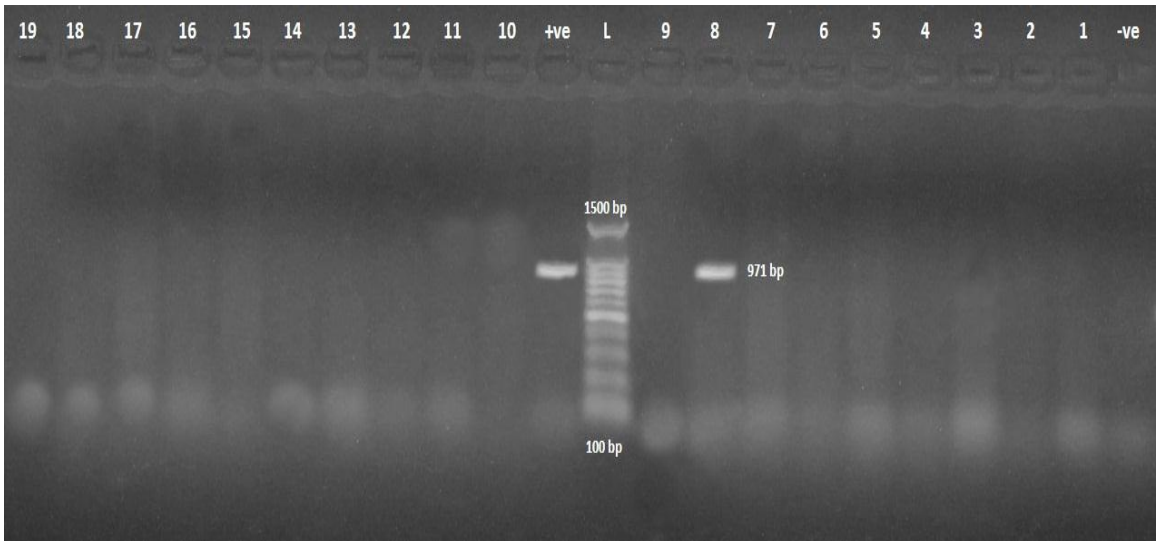

Figure 3. Agarose gel electrophoresis showing the amplification of *bap* at amplicon of 971 bp
Lane (1-7, 9 and 10-19): showed negative samples.
Lane (8): showed positive sample.
L : Molecular size ladder
(+ve): Control positive.
(-ve): Control negative.

Furthermore, Arciola et al. (2002) suggested PCR as molecular tool which have come along side more traditional methods for identification of virulent biofilm-forming strains. The detection of genes governing the production of such extracellular polysaccharide and in particular, the *ica*A and the *ica*D genes, provides a rapid and accurate technique for strain characterization. Vasudevan et al. (2003) suggested that phenotypic and genotypic tests should be used in combination for the determination of biofilm formation in *S. aureus.*

4. Conclusion

The most effective *in vitro* drug for staphylococcal strains isolated from mastitis milk was vancomycin. The use of penicillin for bovine *Staphylococcus*-mastitis is discouraged. The prevalence of MRSA and biofilm producing *S. aureus* isolates from bovine mastitis was high. Molecular techniques like PCR and multiplex PCR are importance and have advantages over the traditional method like CRD for the detection of biofilm production by *S. aureus*.

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