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RESEARCH ARTICLE

Comparison of Biofilm Detection Methods and Antibiogram of Biofilm Positive *S. aureus* on Congo Red Agar

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ABSTRACT

Staphylococcus aureus is a major leading challenge because of biofilm production. Current study demonstrated comparative evaluation of biofilm detection methods and antibiogram of isolates. 79 isolates of *S. aureus* from dairy origin were tested. Standard tissue culture plate method detected 27.78% weak, 27.78% moderate and 5.55% strong biofilm producing *S. aureus* of cattle milk origin and 23.25% weak, 20.93% moderate and 2.32% strong isolates of buffalo milk origin. Tube method revealed 33.33% weak, 30.56% moderate and 8.33% isolates of cattle milk origin with sensitivity 57.14%, 80%, 100% and specificity 50%, 70%, 87.5% respectively and 25.58% weak, 6.98% moderate and 2.32% strong isolates of buffalo milk origin with sensitivity 38.10%, 22.22%, 100% and specificity 83.33%, 90.91%, 100% respectively when compared to standard tissue culture plate method. Congo red agar method detected 38.89% weak, 25% moderate and 19.44% isolates of cattle milk origin with sensitivity 100%, 44.44%, 100% and specificity 38.46%, 55.56%, 50% respectively and 48.83% weak, 30.23% moderate and 2.32% strong isolates of buffalo milk origin with sensitivity 42.86%, 88.89%, 100% and specificity 90.91%, 66.67%, 100% respectively on comparison with tissue culture plate method. Cattle origin isolates revealed that tissue culture plate method detected 60.76%, tube method 72.25% and Congo red agar method 83.54% biofilm positive isolates. The detection sensitivity of tube method and Congo red agar method were 73.68%, 72.22% and specificity 65.63%, 46.88% respectively when compared to tissue culture plate method. Buffalo origin isolates showed that tissue culture plate method detected 72.15%, tube method 35.44% and Congo red agar method 55.70% biofilm positive isolates. The detection sensitivity of tube method and Congo red agar method were 35.48%, 90.91% and specificity 58.06%, 83.33% respectively when compared to tissue culture plate method in buffalo. Statistically, overall biofilm identification methods of buffalo origin isolates are significant. Antibiogram on Congo red agar showed lowest resistance to oxacillin and cefoxitin. Tissue culture plate method is standard method for biofilm detection. Antibiogram revealed oxacillin and cefoxitin sensitivity while amikacin and vancomycin inclined towards resistive pattern. This indicates alarming threat for clinical challenge of biofilm producing *S. aureus*.

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INTRODUCTION

S. aureus is leading food borne pathogen and a great threat to human health (Derebe Tessema and Solomon Tsegaye, 2017)). It is isolated from many food articles but its main entrance point in human food chain is milk (Sakwinska *et al.,* 2011). *S. aureus* resistance to many antibiotics and toxin production capabilities make the situation devastated (Basanisi *et al.,* 2017). Along with, *S. aureus* zoonotic aspect is of major concern now a days (Lee, 2003). Emerging discrepancies and evolution of hyper virulent strains of *S. aureus* are great risks to public health (Kateete *et al.,* 2011; Aqib *et al.,* 2018). About 6% novelty in genome of recent isolates as compared to published genomes of *S. aureus* shows high rate of adoptive evolution in *S. aureus* leading to major challenges in clinics (Holden *et al.,* 2004). In such higher virulence and discrepancies of *S. aureus*, a major point of concern is biofilm production capabilities (Cha *et al.,* 2013). Microbial biofilms and persistence in adoptions in adhering behaviors are constant challenges in medical field as these are difficult to eradicate by antibiotics (Kadurugamuwa *et al.,* 2003). Antibiotic resistance is listed as major global issue by world health organization (Marshall and Levy, 2011). Biofilm production capabilities make pathogen resistant against antibiotics, disinfectants and host immune cells (Stepanović *et al.,* 2007). Biofilm provides protection to *S. aureus* against host immune system, antibiotic treatment, inhibitory factors and enhances colonization, proliferation and persistence (Schilcher *et al.,* 2016). *S. aureus* virulence genes, their pathways, expression systems, phenotypic expressions, coding proteins, their receptors and their association with human diseases are already established (Jarraud *et al.,* 2002). Genes involved in biofilm production, matrix typing and antibiotic resistance of *S. aureus* are correlated (Boles *et al.,* 2010). Biofilm sheath formation in *S. aureus* is dependent on several parameters such as media composition, presence of antimicrobials, pathogen type, temperature, pH, oxygen availability, gene expression pathways, inoculum quantity, hydrodynamic forces and substrate characteristics (Naves *et al.,* 2008). Major role of biofilm matrix components PNAG polysaccharide and extracellular DNA is evident in *S. aureus* resistive nature indicating elicit target for biofilm inhibition (Izano *et al.,* 2008). Protein A is essential part of biofilm matrix which provides aggregation medium in standing and flowing conditions leading to enhancement of biofilm production and sustainment (Merino *et al.,* 2009). Most of multidrug resistant *S. aureus* show higher biofilm production potential without any clonal dispersion (Velázquez-Guadarrama *et al.,* 2017). So, it is the dire need of time to investigate the formation of biofilm and its relation with changing response to antibiotics by methicillin resistant *Staphylococcus aureus* (Cha *et al.,* 2013). It was observed that biofilm formation and strength is dependent on medium used, protocol followed, analysis formulae and bacterial strains (Naves *et al.,* 2008). Several methods are applied for harvesting and investigation of biofilms including tube method, titration plate method, Congo red agar, etc. but there is no standard protocol (Stepanović *et al.,* 2007). That's why, current study was planned to evaluate comparative efficiency of these methods for identification of biofilm production potential of *S. aureus* and antibiotic response of isolates.

MATERIALS AND METHODS

Harvesting of bacterial strains: *S. aureus* strains were isolated from dairy milk and confirmed according to guidelines of (Bergey and Holt, 1994; Aqib *et al.,* 2017).

Biofilm potential evaluation: Biofilm production was evaluated by using Tube method, Tissue culture plate method, Congo red agar method (Hassan *et al.,* 2011).

Tube method: In this method, tryptic soy broth (TSB) seeded with microbial culture was incubated overnight at 37 °C. After washing and staining, visible film lining the wall and bottom of tube was noticed at the end of assay. Intensity of film produced was characterize as 1 (weak/ no biofilm producer), 2 (moderate biofilm producer), and 3 (strong biofilm producer).

Tissue culture plate method: The method involves incubation of TSB seeded with inoculum in 96 well plate. Detection of biofilm is based on optical density calculated at 570nm and graded on the basis of optical density (OD) value, adherence and biofilm formation. OD value < 0.12 showed no adherence and no/ weak biofilm formation. OD value 0.12-0.24 showed moderate adherence and moderate biofilm formation. While OD value > 0.24 showed strong adherence and high biofilm formation.

Congo red agar method: Third method includes incubation of *S. aureus* on Congo red agar (CRA) for 24 h at 35°C. Positive results were indicated by black colonies with dry crystalline appearance. Weak biofilm producers usually remain pink. Black colonies without dry crystalline appearance are indicative of moderate biofilm production.

Antibiotic sensitivity assay: This was done according to guidelines of Clinical and Laboratory

standards 2015 (Institute, 2015). Diffusion media used was Congo red enriched Mueller Hinton agar as it enhances biofilm expression of isolates (Freeman *et al.,* 1989; Amrutha *et al.,* 2017).

Empirical comparison of zone of inhibitions (mm) of susceptible strains of BPSA: To get estimation of capacity of antibiotics to produce zone of inhibitions, statistically ANOVA and t-test were applied on mean of diameters of zones produced by sensitive, intermediate and resistant strains of different antibiotics. To do this, all zones of inhibitions within each antibiotic susceptible category were averaged and compared within each between cattle and buffalo based BPSA, and among sensitive, intermediate, and resistant strains of BPSA within species of animal.

RESULTS

Comparisons of identification methods for BPSA: The study found significant difference ($p<0.05$) of tube method, Congo red agar method, and tissue culture plate method for identification of biofilm production in *S. aureus* isolated from cattle and buffalo milk samples. The identification of biofilm was noted higher (83.54%) when identified by Congo red agar against cattle-based *S. aureus* while tissue culture plate method presented higher (72.15%) in case of buffalo milk-based samples. Sensitivities (%) of identification methods were higher than to their specificities. Current study found higher sensitivity and specificity of tube method than to CRA in cattle milk-based samples while vice versa was noted in case of buffalo milk-based samples (Table 1).

Sensitivity and specificity comparisons for weak, moderate, and strong BPSA identification: There was highly significant difference ($p<0.01$) among weak, moderate and strong biofilm producing *S. aureus* strains identification by each of Tube method and Congo red agar method as compared to tissue culture plate method from both cattle and buffalo milk-based samples. By each method, higher prevalence of weak BPSA was found following which were moderate and strong BPSA from buffalo and cattle milk-based samples (table 2, 3).

Cattle milk based BPSA: The study noted quite variable response of sensitivity and specificity pattern for cattle milk based BPSA than to that of buffalo milk based BPSA identification. The statistical inference showed non-significant difference ($p>0.05$) of methods of BPSA identification for weak BPSA, moderate BPSA, and strong BPSA. Sensitivity comparison between tube method and CRA method for weak BPSA identification showed higher percentage in later (57% versus 100%) while for moderate BPSA identification it was inverse response (80% versus 44.44%), however 100% sensitivity of both methods was noted in strong BPSA identification. Comparison of these two methods for specificities with weak BPSA, moderate BPSA, and strong BPSA showed reduced response in CRA method of biofilm identification in *S. aureus*. Sensitivity and specificity comparisons within tube method showed reduced specificity for each of weak BPSA, moderate BPSA and strong BPSA. While in case of CRA method, moderate BPSA was noted with higher specificity than to sensitivity but the case was inverse in case when weak BPSA and strong BPSA were identified (Table 2).

Buffalo milk based BPSA: Sensitivity of the tube method was higher than that of CRA method both for weak BPSA and moderate BPSA while that of strong BPSA remained same. The specificity of CRA method was found lower than that of tube method for identification of weak BPSA, moderate BPSA and strong BPSA. The sensitivity comparisons with specificities, taking CRA method in account, revealed lower specificity for moderate BPSA identification which was inverse in case of weak BPSA (Table 3).

Empirical analysis of zone of inhibitions produced by BPSA isolates: Another comparison of average zones of inhibitions shown by BPSA of cattle and buffalo in each of sensitive, resistant, and intermediate susceptible categories of different antibiotics were made to present empirical idea of biofilm producing *S. aureus*. The statistical analysis inferred significant difference ($p<0.05$) among ZOIs produced by antibiotics against buffalo BPSAs from sensitive while cattle BPSAs from intermediate category. On the other hands, non-significant difference ($p>0.05$) of ZOIs produced by different antibiotics was noted against cattle BPSA of sensitive and intermediate categories. Highest zones within sensitive strain category of BPSA (buffalo milk) were found against cefoxitin followed by cattle BPSA against oxacillin. The lowest ZOI (20 ± 2.00) was noted against vancomycin in sensitive category. In case of intermediate strains, cefoxitin against buffalo BPSA followed the amikacin intermediate of cattle BPSA to create higher ZOIs. Amikacin produced comparatively higher ZOIs when applied on buffalo milk based BPSA while vancomycin followed amikacin to inhibit growth of both cattle and buffalo based BPSAs (Table 4, 5).

Comparisons between cattle and buffalo BPSA within each category represented no statistical inferences for intermediate strains of all tested antibiotics because of unavailability of relative isolates within the category (fig 1, a). However, there were significant difference of ZOIs between cefoxitin sensitive BPSA of cattle and buffalo origin. On the other hands, non- significant difference

(*P*>0.05) of ZOIs between cattle and buffalo based BPSA of oxacillin sensitive and similarly the amikacin sensitive strains were noted. Vancomycin sensitive strains of cattle based BPSA were not found in the study, so no comparison was practicable (Fig. 1, b). Regarding resistant strains of different antibiotics, only difference that was statistically measurable appeared to be non-significant (*P*>0.05) (Fig. 1c).

DISCUSSION

Biofilm production has been noticed in *Staphylococcus* species and is a point of great concern (Stepanović *et al.*, 2007; Boles *et al.*, 2010; Cha *et al.*, 2013). Investigators have focused on biofilm detection and its composition (Shanks *et al.*, 2005; Kaplan *et al.*, 2012). Under consideration, tube method, tissue culture plate method (TCP) and Congo red agar method (CRA) were mostly used screening procedures (Branda *et al.*, 2005; Merritt *et al.*, 2005). We have tested 79 *S. aureus* isolates for their biofilm production capabilities at different level of potential (weak, moderate, strong). Tissue culture plate method is the golden standard method for detection of biofilm (Christensen *et al.*, 1985; Hassan *et al.*, 2011). In our study, 60.76 % of cattle milk based BPSA and 72.15% of buffalo milk based BPSA were detected positive for biofilm production.

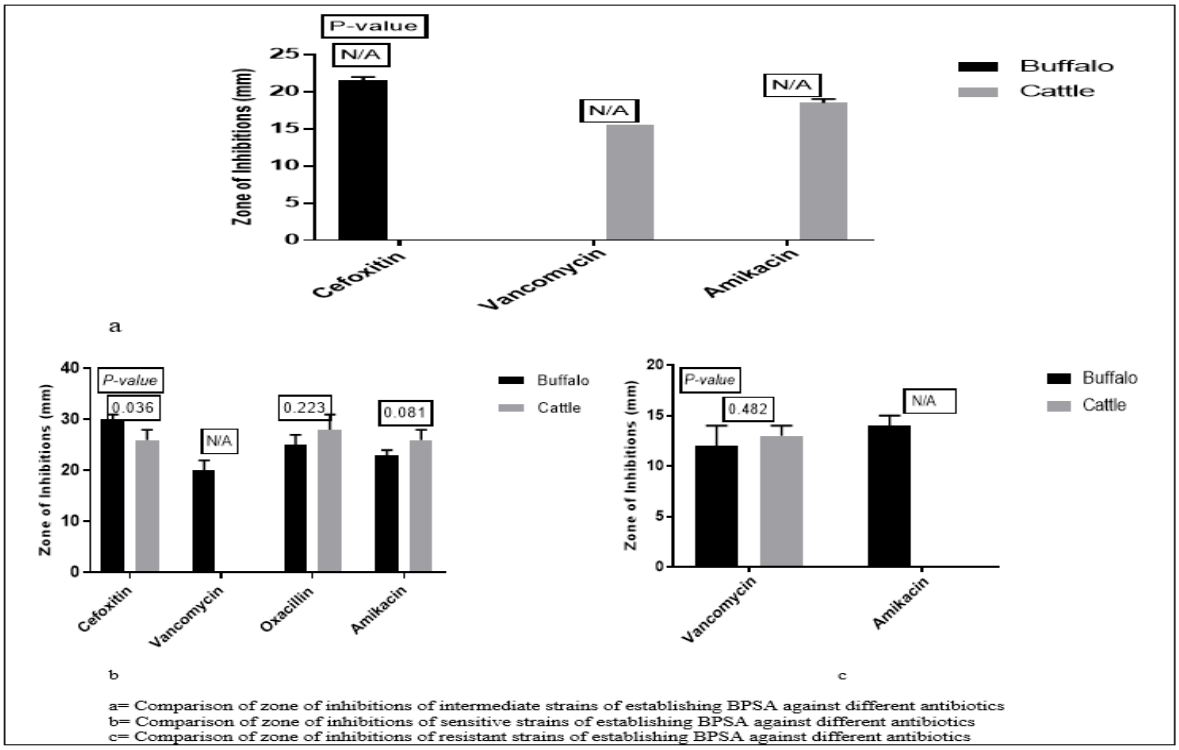

Fig. 1: Comparisons of zone of inhibitions (mm) of establishing BPSA produced by different antibiotics falling in sensitive, intermediate, and resistant categories

Table 1: Overall biofilm production identification by different methods

****Methods**	**Total**	***Overall biofilm identified (Cattle)**					***Overall biofilm identified (Buffalo)**					***p-value* Overall**
		(%)	**CI (95%)**	***P-value***	**Sensitivity (%)**	**Specificity (%)**	**(%)**	**CI (95%)**	***P-value***	**Sensitivity (%)**	**Specificity (%)**	
Tube Method	79	72.15	0.6142 - 0.8083	0.006	73.68	65.63	35.44	0.2579 - 0.4644	0.000	35.48	58.06	0.000
CRA	79	83.54	0.7385 - 0.9012		72.22	46.88	55.70	0.4474 - 0.6614		90.91	83.33	0.000
TCP	79	60.76	0.4973 - 0.7079		-	-	72.15	0.6142 - 0.8083		-	-	0.129

Table 2: Prevalence of biofilm producing *S. aureus* and type of biofilm as identified by different methods (Cattle milk)

**Methods	Total	*Weak Biofilm (%)	CI (95%)	*p-value*	Sensitivity %	Specificity %	Moderate Biofilm (%)	CI (95%)	*P-value*	Sensitivity %	Specificity %	Strong Biofilm (%)	CI (95%)	*P-value*	Sensitivity %	Specificity %
Tube Method	36	33.33	0.2021 - 0.4966	0.607	57.14	50	30.56	0.1801 - 0.4686	0.871	80	70	8.33	0.0287 - 0.2182	0.140	100	87.5
CRA	36	38.89	0.2479 - 0.5514		100	38.46	25.00	0.1375 - 0.4107		44.44	55.56	19.44	0.0975 - 0.3502		100	50.0
TCP	36	27.78	0.1585 - 0.4399		-	-	27.78	0.1585 - 0.4399		-	-	5.55	0.0154 - 0.1815		-	-

P<0.05 indicates a significant difference. ***P*-values in the table show the difference of BPSA among different methods for weak, moderate, strong, and overall basis biofilm identified and non-identified. **P*-values of BPSA comparison between weak, moderate, and strong biofilm within the method were the same for all *P*<0.001

Table 3: Prevalence of biofilm producing *S. aureus* and type of biofilm as identified by different methods (Buffalo milk)

**Methods	Total	*Weak Biofilm (%)	CI (95%)	*p-value*	Sensitivity %	Specificity %	Moderate Biofilm (%)	CI (95%)	*P-value*	Sensitivity %	Specificity	Strong Biofilm (%)	CI (95%)	*P-value*	Sensitivity %	Sensitivity %
Tube Method	43	25.58	0.1493 - 0.4024	0.013	38.10	83.33	6.98	0.0240 - 0.1861	0.019	22.22	90.91	2.32	0.0041 - 0.1207	1.00	100	100
CRA	43	48.83	0.3462 - 0.6325		42.86	90.91	30.23	0.1860 - 0.4510		88.89	66.67	2.32	0.0041 - 0.1207		100	100
TCP	43	23.25	0.1316 - 0.3775		-	-	20.93	0.1142 - 0.3521		-	-	2.32	0.0041 - 0.1207		-	-

P<0.05 indicates a significant difference. ***P*-values in the table show the difference of BPSA among different methods for weak, moderate, strong, and overall basis biofilm identified and non-identified. **P*-values of BPSA comparison between weak, moderate, and strong biofilm within the method were the same for all *p*<0.001.

Table 4: Comparison of zones of inhibitions (mm) produced by establishing BPSA against different antibiotics

Antibiotic	Sensitive		Intermediate		Resistant	
	Buffalo	Cattle	Buffalo	Cattle	Buffalo	Cattle
Cefoxitin	30 ± 1.00	26 ± 2.00	21.5 ± 0.5	N/A	N/A	N/A
Vancomycin	20 ± 2.00	N/A	N/A	15.5 ± 0.5	12 ± 2.00	13 ± 1.00
Oxacillin	25 ± 2.00	28 ± 3.00	N/A	N/A	N/A	N/A
Amikacin	23 ± 1.00	26 ± 2.00	N/A	18.5 ± 0.5	14 ± 1.00	N/A
P-value (ANOVA)	0.000	0.531	N/A	0.002	0.196	N/A

Table 5: Antimicrobial assay on establishing biofilm S. aureus (cattle and buffalo based) using Congo Red agar as growth medium

Antibiotic	Establishing biofilm (Cattle-based)				Establishing biofilm (buffalo-based)				Establishing biofilm (Overall (Buffalo +Cattle)			
	Sensitive %	Intermediate %	Resistant %	p-value	Sensitive %	Intermediate %	Resistant %	p-value	Sensitive %	Intermediate %	Resistant %	p-value
Cefoxitin	(10/10) 100	(0/10) 0.00	(0/10) 0.00	0.000	(7/10) 70.0	(3/10) 30.0	(0/10) 0.00	0.004	(17/20) 85.0	(3/20) 15.0	(0/20) 0.00	0.000
Vancomycin	(0/10) 0.00	(7/10) 70.0	(3/10) 30.0	0.004	(3/10) 30.0	(0/10) 0.00	(7/10) 70.0	0.004	(3/20) 15.0	(7/20) 35.0	(10/20) 50.0	0.062
Oxacillin	(10/10) 100	(0/10) 0.00	(0/10) 0.00	0.000	(10/10) 100.0	(0/10) 0.00	(0/10) 0.00	0.000	(20/20) 100	(0/20) 0.00	(0/20) 0.00	0.000
Amikacin	(7/10) 70.0	(3/10) 30.0	(0/10) 0.00	0.004	(3/10) 30.0	(0/10) 0.00	(7/10) 70.0	0.004	(10/20) 50	(3/20) 15.0	(7/20) 35.0	0.062
P-value	0.000	0.001	0.021	-	0.003	0.021	0.000	-	0.000	0.028	0.000	-

These results are co- in line with observations of (Mathur *et al.*, 2006; Hassan *et al.*, 2011; Cha *et al.*, 2013) as they reported 53%, 63% and 68.35% biofilm positive *S. aureus* detected by TCP method. Somewhat higher percentage in our study could be due to emerging discrepancies and high resistive behavior of *S. aureus* (Aqib *et al.*, 2018).

With tube method, we found 72.15 % cattle milk based BPSA and 35.44 % buffalo milk based BPSA positive for biofilm production with sensitivity

73.68%, 35.48 % and specificity 65.63 % and 58.06% respectively. These results are in agreement to the studies of (Mathur *et al.,* 2006). They observed 78 % and 60 % of *S. aureus* positive for biofilm with 73.6 % sensitivity using tube method. Using CRA method, 83.54 % cattle milk based BPSA and 55.70 % buffalo milk based BPSA positive for biofilm production with sensitivity 72.22 %, 90.91 % and specificity 46.88 % and 83.33 % respectively. Similar observations had been reported by (Mathur *et al.,* 2006; Hassan *et al.,* 2011; Melo *et al.,* 2013) which reported CRA as a method with more than 80% specificity.

On comparing tube method and CRA with standard TCP method, well aligned results of both described methods were observed for detection of strong biofilm (sensitivity 100% for both cattle and buffalo milk based BPSA) but these outcomes were found variable in detection of moderate (Sensitivity 80 %, 44.44 % for cattle milk based BPSA and 22.22 %, 88.89 % for buffalo milk based BPSA) and weak biofilm (57.14 %, 100 % for cattle milk based BPSA and 38.10 %, 42.86 % for buffalo milk based BPSA). These observations are supported by previously reported literature in which tube method was able to show 73% sensitivity and CRA showed lower credit (41 %) (Hassan *et al.,* 2011). Overall variability of biofilm detection rates, CRA cannot be designated as highly valid method for detection of biofilm as compared to tube method and TCP. Tube method was found credible for detection of strong and moderate biofilm producing *S. aureus* but it's difficult to achieve higher accuracy for detection of weak biofilm. These results are in line with (Růžička *et al.,* 2004) who reported that out of 179 isolates, tube method detected 53.7 % and CRA 43.5 % as biofilm positive *Staphylococci* with sensitivity 73% and 11 % respectively. Melo *et al.,* (2013) reported tube method and CRA as screening method for biofilm production capabilities of isolates.

We found higher sensitivity of isolates to cefoxitin and oxacillin (85%, 100% respectively) when Congo red agar was used as growth medium. CRA medium enhances biofilm expression of isolates (Freeman *et al.,* 1989; Rajkumar *et al.,* 2016). These observations were similar to (Broekema *et al.,* 2009; Kumar *et al.,* 2013) who reported cefoxitin and oxacillin sensitive *S. aureus* isolates. In our study, isolates exhibited more resistance to vancomycin (15% sensitive, 35% intermediate, 50% resistant). This pattern was same as observed by (Frank *et al.,* 2007) who reported only 7% isolates sensitive to vancomycin. A study also reported prevalent vancomycin resistance in microbial isolates (Gousia *et al.,* 2015). Amikacin susceptibility shown by *S. aureus* isolates, in our study, was more inclined towards sensitive pattern (50%) than intermediate (15%) and resistant (35%). This is in agreement with studies of (Freitas *et al.,* 1999; Ghazi *et al.,* 2017) which reported high sensitivity of amikacin for resistant isolates. Another study also reported higher susceptibility of aminoglycosides against *S. aureus* (Feng *et al.,* 2016).

CONCLUSION

We found higher percentage of biofilm producing *S. aureus* from dairy origin emphasizing on need of improvement in sanitary practices. Tissue culture plate method is standard method for biofilm detection while other two are capable of detecting strong biofilm at optimal level but give variable results for moderate and weak biofilm detection. Thus, both can be used as screening methods. Antibiogram revealed oxacillin and cefoxitin sensitivity while amikacin and vancomycin inclined towards resistive pattern. This indicates alarming threat for clinical challenge of biofilm producing *S. aureus* in future.

Authors' Contribution

MAN did research, make draft, prepare manuscript, MIS conceive idea, revised manuscript, supervise, AM revised manuscript, IM did analysis and revised manuscript, MS revised manuscript and conceive idea.

Conflict of Interest: The authors declare no conflict of interest.

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