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Phenotypic and Genotypic Detection of Zoonotic Foodborne Pathogens from Dairy Environment and Raw Milk with Special Reference to Biofilm Production

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Abstract

Milk and milk products can harbour multiple varieties of foodborne pathogens, such as *Staphylococcus aureus*, *Shigella* like toxin producing *E. coli*, *Salmonella* and *Bacillus cereus*. Many food-borne outbreaks have been associated with dairy products as main vehicles for transmission. In this study, a total of (n=310) samples were collected from different places dairies, gaushalsa, local shops and vendors of Mathura region. Swabs were taken from milking buckets (50), dippers (64), canes (56) and raw milk (140) from cows. Samples were screened for *S. aureus* and Shiga toxin producing *E. coli* (STEC) by streaking on selective agar and molecularly characterized for housekeeping *nuc* and s*tx* gene by m-PCR. Overall prevalence of *S. aureus* and STEC was revealed 80.64% and 7.41%, respectively. Confirmed *S. aureus* and STEC isolates were screened for biofilm formation capability by phenotypic method methods *viz* Congo red agar (CRA) assay, Tube Method (TM) and Tissue Culture Plate method (TCP). On CRA 9.2 % isolates of *S. aureus* were positive and 90.8% were negative while TM revealed 79.2%, 12.4% and 8.4% strong, moderate and weak biofilm formers. In TCP method, 91.6% isolates were strong, 5.6% moderate and 2.8% weak biofilm producers. Among STEC isolates, 34.78 % and65.22% were positive and negative on CRA while by TM, 43.47%, 26.08% and 30.43% were strong, moderate and weak biofilm formers. In another TCP assay, 52.17%, 30.43% and 17.39 % isolates were strong, moderate and weak biofilm producers, respectively. Among these three methods TCP was found more sensitive for *S aureus* as well as STEC. Under Scanning electron microscopy, the 3D structure of biofilms of *S. aureus* and STEC revealed and the biofilms were well organized, with intact cell-to-cell connections. STEC produced better biofilm than *S aureus*. This study revealed that biofilm forming *S. aureus* and STEC were obtained from dairy utensils and raw milk so, may be a sustainable source of contamination of dairy products. So, there is need of paying more attention to the cleaning and sanitizing processes of food contact surfaces to ensure the public health.

Key words: STEC, *S. aureus*, CRA assay, TCP, SEM, Tube Method

Introduction:

The key task of dairy industry is the production of quality and safe milk and milk products and the main factor that reduces storage life and safety of dairy products is the micro-organisms (Alghizzi and Shami, 2021). Food safety presents a significant challenge to global social and economic advancement, particularly in less developed countries (Oriekhoe et al., 2024). Food borne pathogen like *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium, and Listeria monocytogenes*, contaminate different foods like dairy products, handmade goods, meat and vegetables and cause danger to human health (Shi et al., 2024). Shiga toxin producing *E. coli* (STEC) is a subtype of *E. coli* that causes enteric and systemic diseases ranging from diarrhoea to severe haemorrhagic colitis (HC) hemolytic uremic syndrome (HUS) and Thrombocytopenic purpura (TPP) (Parul et al., 2021).

Quantitative and qualitative composition of microflora of the products depends on the compliance with hygienic conditions of production and effective sanitation of technological equipment (Cui et al., 2020). The most significant source of microbial contamination of food products during production is technological equipments and about 40% of the food poisoning of people in the world is caused by microorganisms that penetrate raw materials and finished products from processing equipment (Regasa et al., 2019). Microflora mostly survives on the surfaces of equipment during sanitation so-called “dead zones” (bends, joints, gaskets, valves, cracks, scratches) due to the formation of a biofilm (Aliyu et al., 2020).

Biofilm-forming bacteria are known to be a major source of both spoilage and pathogenic microflora in the dairy industry (Bai et al., 2023). Therefore, bacteria that form biofilms may adversely affect the safety and quality of milk and its products. The main source of contamination of dairy products is often associated with the formation of biofilms on the surfaces of milk transport pipes, milking containers, and accessories in the dairy industries (El-Far

et al., 2021). The bacteria may detach from biofilms and contaminate the milk as it passes surfaces. Many attempts have been made to combat biofilm formation in the dairy industry, including cleaning and thoroughly disinfecting surfaces that are exposed to milk during its processing (Tibeu et al., 2021). However, antimicrobial treatment may be compromised since the disinfectants do not penetrate the biofilm's matrix that is mounted on the surface (Yadav et al., 2015). According to research studies the equipment on which at least one plankton bacteria was detected carries about 1,000 microorganisms formed in the biofilms (Yuan et al., 2020).

Data from the scientific literature indicate that microbial biofilms protect bacteria during sanitization and help to survive on equipment. That is why; it poses a great risk to safety, security and quality of dairy products and to the health of consumers who get exposed to such products. Therefore, a detailed study was designed to study foodborne zoonotic pathogens Isolated from Dairy Environment and Raw Milk with special reference to biofilm production.

Materials and Methods:

Sampling

A total of 310 samples comprising of retail milking buckets (n=50), dippers (n=64), milking canes (n=56) and raw milk (n=140) were collected from dairies, gaushalas, local shops and vendors in nearby regions of Mathura district of Uttar Pradesh, India. The samples were brought in chilled condition and processed within 24 hrs for the isolation and identification of *S. aureus* and *E. coli* was done as per the standard microbiological techniques (Kou et al., 2021) and Edwards and Ewing (1972) with slight modifications, respectively.

Isolation and identification of *S. aureus* and *E. coli*

For the isolation of *S. aureus* the samples of milk (1ml) and utensils swabs were enriched in 9 ml and 5 ml of buffered peptone water (BPW) at 37°C for 24 hrs, respectively. The loopful culture growth from BPW was streaked on Baird Parker agar and incubated at 37°C for 24 hrs. *Staphylococcus spp*. produced peculiar jet-black colored colonies over this agar and single jet-black colored colony per positive sample was picked and streaked on mannitol salt agar (MSA) and incubated at 37°C for 24 hrs. On MSA, staphylococci produced golden yellow colonies surrounded by yellow zone and single colony from MSA was picked and streaked on nutrient agar slant and incubated at 37°C for 24 hrs and stored at 4°C till further processing.

The isolation and identification of *E. coli* was done as per the method given by Edwards and Ewing (1972). The samples of milk (1ml) and utensil swabs were enriched in 9 ml and 5 ml of Tryptone soya broth at 37°C for 24 hrs, respectively. The loopful culture growth from TSB was streaked on MacConkey lactose agar and incubated at 37°C for 24 hrs. Lactose fermenting pink-colored colonies were picked and streaked over Eosin methylene blue (EMB) at incubated at 37°C for 24 hrs. The colonies showing green metallic sheen were presumptive *E. coli* and subjected for biochemical confirmation. Single colony of *E. coli* was taken from each positive sample.

Molecular detection of housekeeping genes

After biochemical confirmation, *S. aureus* was further subjected to monoplex PCR for screening of housekeeping *nuc* gene as per the procedure given by Brakstad et al. (1992) and details of primer were mentioned in Table 1. STEC were further subjected to multiplex PCR for screening of housekeeping Shiga toxin like gene as per the protocol given by Paton and Paton (1998) and details of primer were mentioned in Table 1**.** PCR was carried out in a final reaction volume of 25 µl containing 12.5 µl of Master mix, 0.25 µl of each of the primers (forward and reverse) for each gene, DNase free water 8.5 µl and finally 2.0 µl of DNA template was added and PCR reaction was performed in a thermal cycler (Cyber lab) using standard cycling condition.

Biofilm production by phenotypic assays

S. aureus and STEC isolates were observed for the biofilm forming capacity *in vitro* by three different assays *viz.* Congo red agar (CRA) assay, Tube method (TM) and Tissue culture plate (TCP) assay. In CRA assay, black colored colonies with a dry crystalline consistency on CR agar were indicate biofilm producers, whereas colonies showing red color were considered non-biofilm producers (Panda et al., 2016). In TM, visible film lined in the wall and bottom of the tube were considered as positive and strong biofilm former (Christensen et al., 1985). In TCP assay, OD values were considered as an index of bacteria adhering to the surface and forming biofilms. Strains were classified into three categories: weak biofilm producers, when ODc< OD ≤ (2 ×ODc), moderate biofilm producers, when (2 ×ODc) < OD ≤ (4 ×ODc), and strong biofilm producers, when (4 ×ODc) < OD (Rodriguez - Lazaro et al., 2018).

Screening of biofilm forming gene

Biofilm forming *S. aureus* was screened for biofilm forming *bap* (biofilm associated protein) gene by monoplex PCR according to Cucarella et al. (2004) and primer details were depicted in Table 1.

Scanning electron microscopy (SEM)

SEM was performed to observe 3D structure of biofilms of *S. aureus* and STEC. Firstly, single colony was used to inoculate in 5 ml of TSB and incubated at 37°C for 12 hrs. Bacterial culture with matched with 0.5 McFarland

and further diluted to 100 times having the range of bacterial cells in between 10^5 to 10^6 cells per ml. In 24 well plates a cover slip of 1 cm diameter was placed at the bottom of wells and each well was filled with 1.8 ml of TSB supplemented with sucrose (1% w/v) and 200 µl of bacterial inoculum and incubated for 24-48 hrs at 37°C. Thereafter, wells were rinsed 3 times with 2 ml of PBS to eliminate non-adherent bacteria. The plate was gently washed thrice with sterile PBS to remove the planktonic cells. Pre-fixing of samples was done by immersion in 2% glutaraldehyde in 0.1 M phosphate buffer. The samples were treated with gradient ethanol (30%, 50%, 70%, 90% and 100%) (Lin et al., 2020). Antibiofilm effect of eugenol was observed under a Scanning electron microscope (JEOL-JSM 6510 LV), at University Sophisticated Instruments Facility (USIF), AMU, Aligarh, Uttar Pradesh, India.

Results and Discussion:

Milk and milk products can harbour a number of spoilage and pathogenic microorganisms including multidrug resistant food borne pathogens. In the last two decades, the presence of foodborne pathogens in foods, including milk and milk products and their biofilm forming capabilities has been reported often in worldwide, raising public health concerns (Picozzi et al., 2017). In order to monitor prevention and control of biofilm forming food borne pathogen it is necessary to understand their sources, the phenotypic and genotypic characteristics and transmission dynamics.

Isolation and identification of *S. aureus* and *E. coli*

A total of 250 presumptive *S. aureus* were isolated from 315 samples (single colony was taken from each positive sample) and phenotypically confirmed by production of jet-black coloured colonies on Baird Parker agar and golden yellow colonies on MSA and by series of biochemical tests. The prevalence of *S. aureus* was found to be 82.0% (41/50), 59.37% (38/64), 76.78% (43/56) and 91.42% (128/140) in milking buckets, milk dipper, milk cane and raw milk, respectively with an overall prevalence of 80.64% (250/310) in all studied sources (Table 2). In raw milk *S. aureus* was 80.64% prevalent results were in contrast with the study of Li et al. (2017) who reported 22.0% in milk of healthy cows from China. Thaker et al. (2013) and Nhatsave et al. (2021) revealed 6.0% and 41.0% prevalence of *S. aureus* in raw milk from Gujarat in India and Mozambique, respectively. Prevalence values were lower to current study. Prevalence of *S. aureus* in pooled raw milk was 57.5% (23/40) collected from vendors and retail milk sellers the outcomes were higher to 34.0% prevalence of *S. aureus* in bulk tank milk from bovine in Greece (Papadolous et al. 2018). In current study, prevalence of *S. aureus* in dairy utensils was 78.23% while in study of Regasa et al. (2019) 20.0 % *S. aureus* isolates were revealed from milking buckets that are quite lower to current study.

Out of 315 samples, 117 samples produced pink colored colony on MLA and metallic sheen on EMB and single colonies was taken from each positive sample. The prevalence of *E. coli* was 60.0% (30/50), 42.18% (27/64), 33.92% (19/56) and 29.28% (41/140) in milking buckets, milk dipper, milk cane and raw milk, respectively with an overall prevalence of 24.43% (117/310) (Table 3). Prevalence of *E. coli* in retail raw milk was 24.43%, almost similar prevalence value of *E. coli*22.4% and 22.2% was revealed from milk in the work of Awadallah et al. (2016) and Ibrahim et al. (2022). Difference in prevalence values of food borne pathogen *S. aureus* and STEC from other studies may be due to difference in geographical condition and processing procedures of samples.

Molecular detection of housekeeping genes

All the phenotypically confirmed isolates were *nuc* gene bearers thus housekeeping gene was present in all the *S. aureus* isolates with prevalence of 100.0% (Figure 1). In the various studies researchers revealed 37.32% and 50.62% *nuc* genes from various milk sources in Turkey and Bangladesh, respectively (Keyvan et al., 2020; Shahid et al., 2021). All the phenotypically detected *E. coli* strains (n=117) were subjected to mPCR, result showed 23 isolates were positive for either *stx*1 or *stx*2 or both the genes (Figure 2) with percent positivity of 19.65% (23/117) and prevalence of STEC was 7.41% (23/310). Mohammadi et al. (2013) revealed 56.41% *stx*2 genes from milk sources in Iran that is higher to this study. Ombarak et al. (2016) revealed 0.9% prevalence of *stx*1gene from Egypt, consistent to this study while in contrast Elafify et al. (2019) revealed 28.8% prevalence of *stx*1gene in Iran.

Biofilm production by phenotypic assays

Biofilm production recognized as an important virulence factor of some of food borne pathogens and a major concern for the dairy industry and is frequently related with lack of monitoring standards and unhygienic practices followed during processing and handling of milk and milk products. Confirmed *S. aureus* and STEC isolates were screened for biofilm formation capability by phenotypic method methods *viz.,* Congo red agar (CRA) assay, Tube Method (TM) and Tissue Culture Plate method (TCP). In CRA method, 9.2 % isolates of *S. aureus* produced black colony and found positive (Figure 3) while 51.6% were found negative on CRA. In TM method, isolates were categorized as 79.2% strong, 12.4% moderate and 8.4% were weak biofilm formers. In TCP method 91.6% isolates were strong followed by 5.6% and 2.8% moderate and weak biofilm producers

(Figure 5). Results of biofilm production are in contrast with Younis et al. (2021), who reported 26.6.0% non-biofilm former and 73.3% biofilm formers. Kou et al. (2021) revealed 66.1% strong 32.3.0% moderate and 1.6% weak biofilm formers by TCP which is lower to the current study.

Among STEC isolates,34.78 % and 65.22% were positive (Figure 4) and negative on CRA while by TM, 43.47%, 26.08% and 30.43% were strong, moderate and weak biofilm formers. In another TCP assay, 52.17%, 30.43% and 17.39 % isolates were strong, moderate and weak biofilm producers, respectively. In the study of Ponnusamy et al. (2012), 37.0% biofilm formers and 63.0 % were non-biofilm formers *E. coli* revealed in CRA assay that is almost consistent to our study. By using the Tube method, total 69.55% STEC isolates showed the ability to produce biofilms. Nosrati et al. (2017) revealed 23.0% strong, 59.0% moderate and 18.0% weak biofilm formers and Nachammai et al. (2016), reported 57.0% biofilm forming *E. coli* from tube method that is lower to current study. In TCP assay, overall, 82.6% isolates showed the ability to produce biofilms (Figure 5) while in the work of Wang et al. (2016), 25.39%, 31.25% and 28.9% were strong, moderate and weak biofilm formers which are lower to this study. The findings of our study suggest that the TCP Plate Assay is a more reliable method for the detection of biofilm forming STEC as compared to TM and CRA method results are consistent with Verma et al. (2023). The discrepancies in the categorization of biofilm phenotypes could result from differences in the interpretation of results thus standardization of the biofilm method is crucial. It has been previously demonstrated that the phenotypic expression of biofilm production, ability is influenced by number of factors including composition of medium also.

Screening of biofilm forming gene in isolates

In this study, none of the *S. aureus* isolates revealed having biofilm forming *bap* gene with prevalence value of 0.0%. In contrast to the current results, other studies showed the *bap* gene occurrence in biofilm forming *S. aureus* isolates was of 10.0% by Ballah et al. (2022). In favour to present study, phenotypically confirmed biofilm former *S. aureus* was negative for *bap* gene was revealed in the study of Notcovich et al. (2018). Similarly, this study also revealed no association between the formation of biofilms on the phenotypic basis, and the presence of *bap* genes in biofilm formation.

Scanning electron microscopy (SEM)

The SEM analyses of the established biofilm of *S. aureus* and STEC revealed that the biofilm was very well organized in 3D structure, with intact cell-to-cell connections (Figure 6a, 6b and 7a, 7b). The results of SEM were in accordance with the studies of researchers, Yadav et al. (2015) and Kim et al. (2016) which revealed the almost similar architecture of *S. aureus* and STEC biofilms.

Conclusions:

The present study concluded that *S. aureus* and STEC have tendency to form biofilms and gets attached to the surface of foods and containers. This leads to an increase in the level of microbial load of food products including dairy products, thus may cause economic losses to the dairy industry. The present study revealed that STEC has more adhering capability on the surfaces as compared to *S. aureus*. The outcome of study might be useful for the control of biofilm forming food borne pathogens.

Conflict of Interest:

Author declared no conflict of interest.

Author's Contribution:

All the authors contributed equally in the preparation of manuscript.

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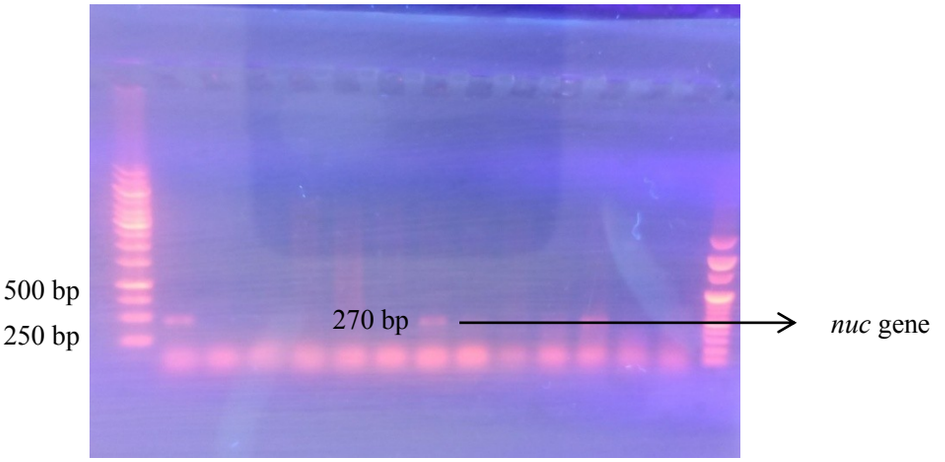

Figure 1: Species Specific PCR amplicon of *S. aureus* resolved after electrophoreses in 1 % agarose Gel
Lane 1: 1kb DNA ladder (bp)
Lane 2: Positive control
Lane (7and11): (*nuc* gene of *S. aureus*of 270bp)

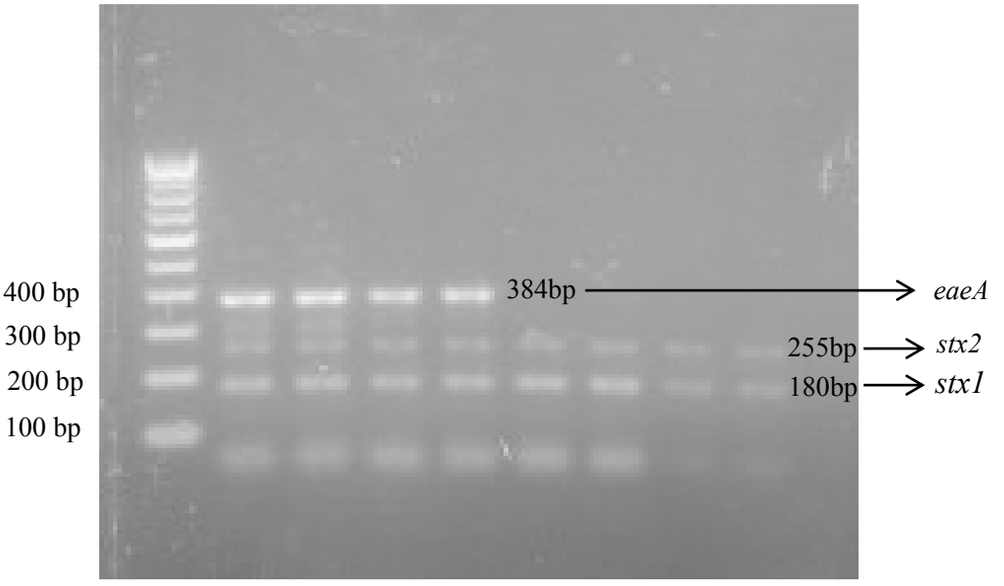

Figure 2: Agarose Gel showing PCR amplified product of virulent gene (s*tx1,stx2,eaeA*) of STEC in different combinations
Lane 1: 100bp DNA ladder,
Lane 2 to 5: s*tx1, stx2, eaeA*geneof STEC
Lane (6 to 9): s*tx1, stx2,* geneof STEC

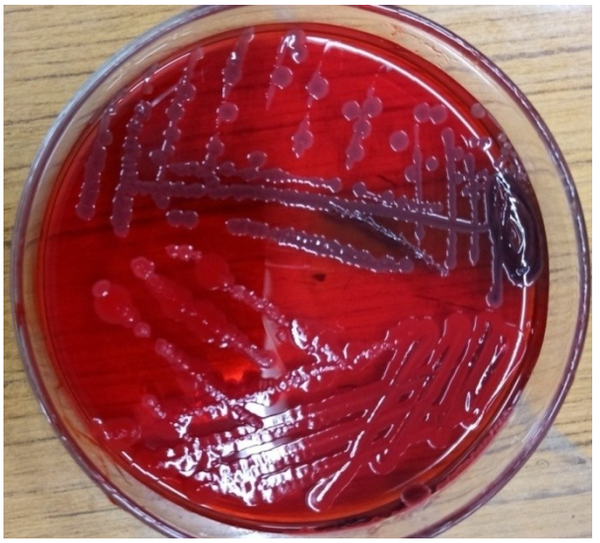

Figure 3: Screening of biofilm producer *S. aureus* by Congo Red Agar assay
Black colonies (+ve for biofilm formation) Red colonies (-ve for biofilm formation)

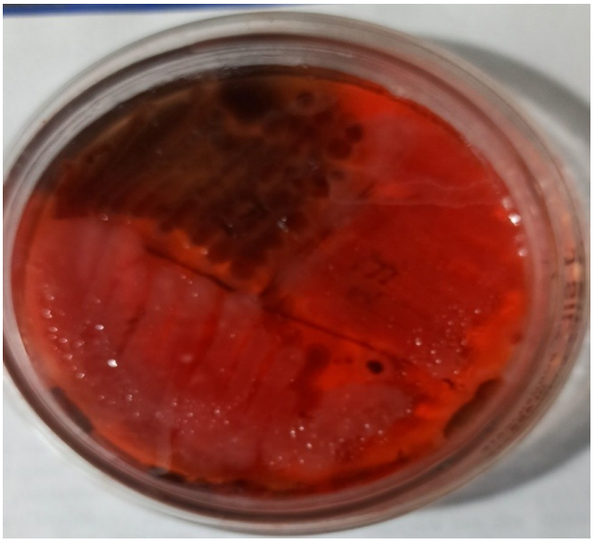

Figure 4: Screening of biofilm producer STEC by Congo Red Agar assay
Black colonies (+ve for biofilm formation) Red colonies (-ve for biofilm formation)

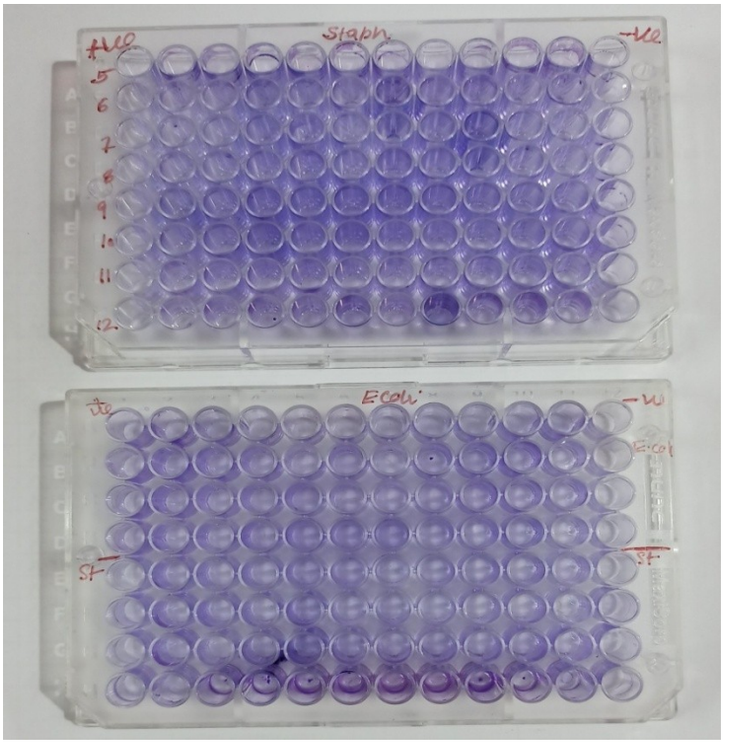

Figure 5: Screening of biofilm producers (*S. aureus*and STEC) by Tissue Culture Plate method

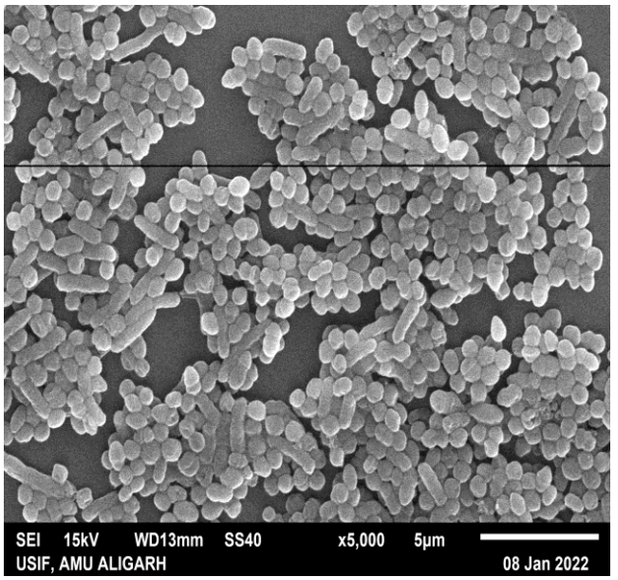

Figure 6a: *S. aureus* biofilm observed by SEM at low magnification (5,000 X)

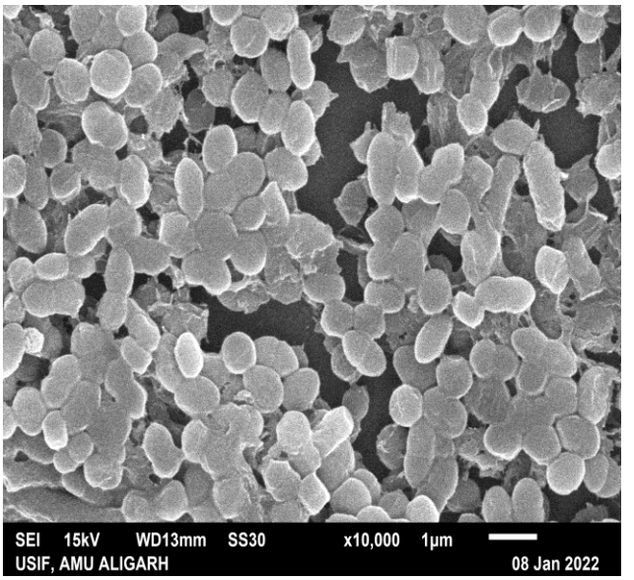

Figure 6b: *S. aureus* biofilm observed by SEM high magnification (10,000X)

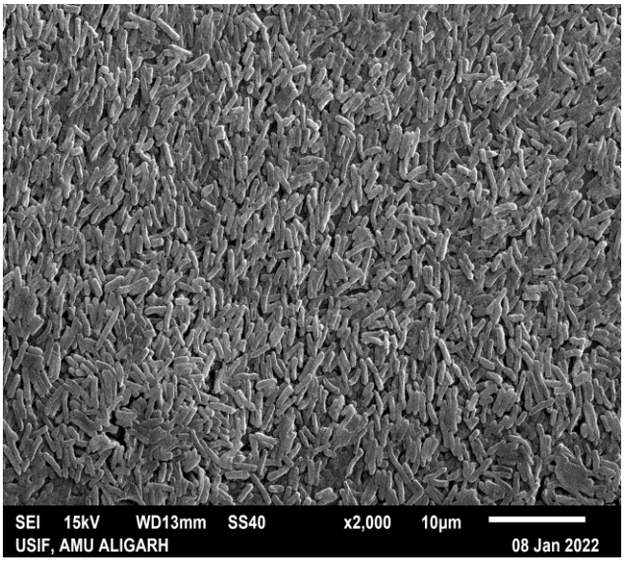

Figure 7a: STEC biofilm observed by SEM at low magnification (2,000 X)

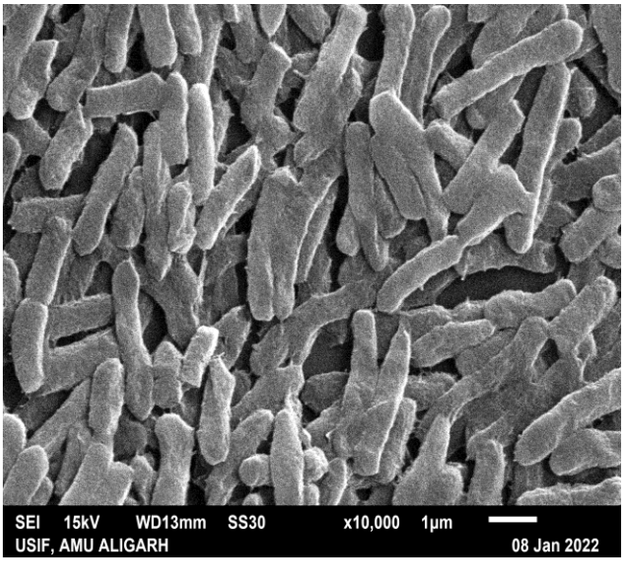

Figure 7b: STEC biofilm observed by SEM at high magnification (10,000 X)

Table 1: Primer details for housekeeping (*nuc*), shiga toxin gene (stx1 and stx2) and biofilm forming (*bap*) genes

S. No.	Targeted Gene (F/R)	Primer sequences	Amplicon size (bp)	References
1.	*nuc* F	5'GCGATTGATGGTGGATACGGTT3'	267	Brakstad et al. (1992)
	nuc R	3'AGCCAAGCCTTGACGAACTAAAGC5'		
2.	*stx*1 F	5'ATAAATCGCCATTCGTTGACTAC3'	180	Paton and Paton, 1998
	*stx*1 R	5'AGAACGCCCACTGAGATCATC3'		
3	*stx*2 F	5' GGCACTGTCTGAAACTGCTCC3'	255	Paton and Paton, 1998
	*stx*2 R	5'TCGCCAGTTATCTGACATTCTG3'		
4.	*bap* F	5'CCCTATATCGAAGGTGTAGAATTGCAC3'	971	Cucarella et al. (2004)
	bap R	5'GCTGTTGAAGTTAATACTGTACCTGC3'		

Table 2: Prevalence of Biofilm Forming *S. aureus* in dairy utensils and raw milk

SN	Source	Total samples tested	No of positive samples (nuc gene)	Prevalence of *S. aureus* (%)	Prevalence of Biofilm forming S aureus TCP method			Prevalence of biofilm forming *S. aureus* (%) TCP	Prevalence of Biofilm forming S aureus TM method			Prevalence of biofilm forming*S. aureus* (%) (TM)	Prevalence of Biofilm forming S aureus CRA	
					S	M	W/N	S+M	S	M	W/N	S+M	P	N
1	Milking Buckets	50	41	82.00 (41/50)	37	3	1	80.0 (40/50)	25	11	5	72.0 (36/50)	08	33
2	Milk Dipper (Measurement)	64	38	59.37 (38/64)	32	4	2	56.25 (36/64)	25	7	6	50.00 (32/64)	2	36
3	Milk Cane	56	43	76.78 (43/56)	37	5	1	75.0 (42/56)	31	5	7	64.28 (36/56)	07	36
4	Raw Milk	140	128	91.42 (128/140)	123	2	3	89.28 (125/140)	117	8	3	89.28 (125/140)	06	122
5	Total	310	250	80.64	229	14	7	78.38 (243/310)	198	31	21	73.87 (229/310)	7.41 (23/310)	73.2 227

Table 3: Prevalence of Biofilm Forming Shiga toxin-producing *E. coli* (STEC) in dairy utensils and raw milk

SN	Source	Total samples tested	Prevalence of *E. coli* (%)	STEC Positive samples	Prevalence of STEC (%)	Prevalence of Biofilm forming *E. coli* TCP method			Prevalence of biofilm forming STEC(%)(TCP)	Prevalence of Biofilm forming STECTM method			Prevalence of biofilm forming STEC (%) (TM)	Prevalence of Biofilm forming STEC CRA	
						S	M	W/N	S+M	S	M	W/N	S+M	P	N
1	Milking Buckets	50	30A	09	18.0 (9/50)	05	03	01	16.0 (8/50)	05	02	02	14.0 (7/50)	03	06
2	Milk Dipper (Measurement)	64	27	04	6.25 (4/64)	02	01	01	4.6 (3/64)	01	01	02	3.1 (2/64)	02	02
3	Milk Cane	56	19	04	7.14 (4/56)	02	01	02	5.3 (3/56)	01	02	01	5.3 (3/56)	01	03
4	Raw Milk	140	41	06	4.28 (6/140)	03	02	02	3.57 (5/140)	03	01	02	2.85 (4/140)	02	04
5	Total	310	117	23	7.41 (23/130)	10	07	04	6.1 (19/310)	10	06	07	5.1 (16/310)	08	15

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