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DECIPHERED ENTITY OF EXTENDED SPECTRUM BETA LACTAMASE RESISTANT *STAPHYLOCOCCUS AUREUS* (ESBL) FROM COWS MASTITIS RAW MILK ECOSYSTEM IN BAGHDAD IRAQ

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Abstract

Stress adaptation cascaded by stress hardening in epigenetic drifted tolerant versus genetic shifted resistant forbidden clones of biofilm producing and multidrug resistant strains of *Staphylococcus aureus* recovered from raw milk of mastitis Cows deciphered an emergent hazard cornerstone policy in public health and food chain. Special topics in molecular epidemiology of phenotypic drift plus genotypic shift coherent pairs of verified clones of *S. aureus* recovered from scanned zones of Abu-Ghraib, Al-Fudhaliyah and Al-Sadrya throughout specified periods represent the critical focus issue of this project. Ninety pooled samples collected from all scanned zones in which, they either, collected from Cows with abnormal milk and shifted behavior or from milk cans deposited in this area. Thirty pooled samples from each zone with periodic cross-linked table throughout January until June (2019). Modified and verified techniques were dependent for recovery module under the guidelines experience of Supervisor (Al-Shammary) with standard reference workstation. IMMUCELL California Mastitis test kit was dependent in segregation pattern of subclinical and clinical mastitis behavior cascaded by total and differential predicted leukocytes counts. Dual modified combo pattern interphase media were dependent in which, enrollment with verified tryptone-soya yeast-extract enriched broth cascaded by culturing module on modified Mannitol salt agar and tri molecular pattern of human, sheep and ox blood agars. Gram and capsule stains was dependent for micrographic features. Confirmation series strategy with catalase, tube coagulase and gelatinase were dependent. Haemolysis pattern, siderophore profile, Staphyloxanthin pigmentation index and modified tissue culture biofilm score were dependent. Antibiogram assays with selected reference antibiotics discs in which, modified Kirby-Bauer disc diffusion method was dependent plus verification of ESβL activity of multidrug resistant clones via Oxoid-Remel Cefpodoxime Combination Kit in which, Cefpodoxime, Cefpodoxime/Clavulanic acid with Ceftazidime were dependent. Frequency and distribution pattern of topic denominator unveiled recovery of nine isolates out of ninety-pooled samples (10 %) in which, five isolates from Abu-Ghraib (5.56 %), two from Al-Sadrya (2.22 %) and two from Al-Fudhaliyah (2.22 %). Checkerboard resistance pattern unveiled five resistant MRSA-VRSA clones (55.56 %), two ESβL clones (22.22 %) from clinical mastitis in Abu-Ghraib, and one AmpC producers (11.11 %) from subclinical cases in Al-Fudhaliyah with one phenotypes (11.11 %) as intermediate to susceptible. In conclusion, deciphered gadget match of recovery module of biofilm producing and multidrug resistant chimeric strains of *S. aureus* from mastitis milk in Baghdad proved as notifiable emergent new epidemiological cases that needs more sophisticated and verified monitoring programs engaged hazard analysis and critical control points like potential bacteriophages in our food chain ecosystem.

Keywords **:** Cows raw milk, Mastitis, Extended Spectrum Beta Lactamase Resistance, *Staphylococcus aureus*.

Introduction

Checkerboard contamination of raw and processed milk and dairy series with forbidden foodborne pathogens and their toxins and enzymes like biofilm producing and multidrug resistant *S. aureus* represent risk policy in our food chain and so on proceeds to our lifestyle (FDA, 2020; CDCs, 2020 and Pal *et al*., 2020). Versatile and diverse distribution pattern of these opportunistic infectious hazard foci with their adaptation plasticity from nosocomial behavior to community our food chain represent dangerous epigenetic and genetic, drift and shift, tolerance and resistance emergent profile that result from stress adaptation cascade by stress hardening phenomenon due to phenotypic action potential module, conjugated forbidden plasmids, transduction with forbidden prophages or transformation by environmental forbidden genetic material (eDNA) (Ferrandon, 2009; Kanaan, 2013; Kanaan and Al-Shammary, 2013; Begley and Hill, 2015; Brauner *et al*., 2016; Bonneaud *et al*., 2019; Reismana *et al*., 2019 and Pal *et al*., 2020). Quorum sensing behavior cascaded by clustered regularly interspaced short palindromic repeats (CRISPR-CAS) defense module and genes sharing strategies with other microbiome in new developed and emergent upgraded strains of *S. aureus* can seriously life threatening cornerstone policy (Rath *et al*., 2015; CDCs, 2020; Medscape, 2020 and Pal *et al*., 2020).

Many of the remaining challenges in infectious disease control involve pathogens that fail to elicit long- lasting immunity in their hosts. Antigenic variation is a common reason for this failure and a contributor to the complexity, and often correctly, invoked to explain antigenic variability in pathogens. However, there is a wide variety of patterns of antigenic variation across space, time within, and between hosts, and we do not yet understand the determinants of these different patterns. This project describes such patterns of drifting and shifting mechanisms in extended spectrum beta lactamase resistant strains (ESβLs) that might occurs during symposia ecosystem interconnected interactions among *S. aureus* with environment, host and other Eco biota. Pathogen-specific explanations for these patterns of diversity are critically evaluated, and the patterns are compared against predictions of theoretical models for antigenic diversity. Major remaining challenges highlighted, including the identification of key protective antigens in study model, the design of vaccines to combat antigenic variability for shifting and drifting like in influenza viruses and the development of more systematic explanations for patterns of antigenic variation (Lipsitch and O'Hagan, 2007 and Pal *et al*., 2020).

Acquisition of foreign set of resistance pattern from Gram-negative bacteria including extended spectrum beta lactamases and carbapenemases rendering them recalcitrant

to treatment remain under theoretical hypothesis unless uncontrolled and unknown mechanisms that occurs shifting them to chimeric strains like what occurs in covid-19 ending with sophisticated hygienic problem that may invade the world with unfortunately forbidden consequences. Our cited research from thesis undergoes burden of recovery module of such developed emergent entity of super forbidden clone of ESβL *S. aureus* from mastitis in Baghdad ecosystem.

Materials and Methods

Authorized guidelines recommended before establishment the main wok design from December 2018 until January 2019 at Milk Hygiene Unit \ Department of Veterinary Public Health. In order to minimizing the laboratory technical errors and to provide, verify and testing different reference procedures in food microbiology about recovery of foodborne denominator ***S. aureus*** specially recalcitrant enrolled encapsulated biofilm and multidrug resistant variants phenotypes (MRSA, VRSA, ESβL, CRISPR-CAS and Chimera) in mastitic milk.

Collection and Processing of Suspected Raw Milk Units: A ninety-pooled samples (five units from each territory per month) collected from different scanned zones post inspection with case history about clinical and subclinical cases of mastitis or from milk cans. Each quarter was stripped first drops of contaminated milk (critically discard first stream of milk), and then collect at least (25-50) ml of infected milk aseptically inside labelled screw-capped containers in which, at least (100-200) ml pooled samples transferred as soon as possible inside icebox to milk lab. Collected samples mixed well then subjected to screening California mastitis test (CMT) and enrolled indirectly on modified combo enriched-enrichment media.

California mastitis test: According to scheduled instructions present in leaflet sheet manual of manufacturing company of IMMUCELL, Portland (USA (2019) the test was don under authorized supervisor announcements. Firstly, food safety cabinet disinfected with ethyl alcohol then collected samples with kit components: a white plastic paddle with four receptacles and diluted CMT anionic surface-active reagent - alkyl aryl sulfonate and an indicator dye - bromcresol purple. About (5-10) ml milk samples collected from each quarter were placed inside four shallow cups of paddle marked A (RH), B (LH), C (LF), and D (RF) to help identify the individual quarter from which the milk was pooled. The CMT solution reconstituted according to package instructions. Added samples quantity should be enough to emerge circles of each receptacle of paddle. Then adding equal amount of CMT reagent to each cup and horizontally swirl for (10-30) seconds until appearance of viscous gel or slim or particles or flakes and clots. reduction speed of purple color to white again due to reaction of CMT reagent specifically with the deoxyribonucleic acid (DNA) of leukocytes specifically polymorphonuclear neutrophils (PMNs) indicating positive score and determining amount of inflammatory cells per ml in milk with udder healthy status, or negative score when milk remain normal with purple - white color. Table (**1**) illustrate gadget match of prediction mastitis chart.

Table 1 : Prediction, Grading and interpretation scoring CMT leukocytes-PMNs mastitis chart (Schalm and Noorlander, 1956 and Coles, 1980).

SCORE	MEANING	DESCRIPTION OF VISIBLE REACTION	INTERPRETATION	INDIVIDUAL QUARTER HEALTHY STATUS
N	Negative	Purple-white Mixture remains liquid with no evidence of formation of a precipitate. No slime or gel form. It can drip out of the paddle well.	0 - 200,000 cells/ml. 0-25 percent PMN.	No Mastitis
T	Trace	A slight precipitate forms and is seen to best advantage by tipping the paddle back and forth and observing the mixture as it flows over the bottom of the cup. Trace reactions tend to disappear with continued movement of the fluid. Mixture becomes slimy or gel like. It's seen to best advantage by tipping paddle back and forth, observing mixture as it flows over the bottom of cups.	150,000 – 500,000 cells/ml. 30-40 percent PMN.	Trace of mastitis Subclinical. Mastitis in one or more quarters
1	Weak Positive	A distinct precipitate but no tendency toward gel formation. With some milks, the reaction is reversible, for with continued movement of the paddle the precipitate may disappear.	400,000 – 1,500,000 cells/ml. 40-60 percent PMN.	Subclinical to clinical Mastitis
2	Distinct Positive	The mixture thickens immediately with some suggestion of gel formation. As the mixture is caused to swirl, it tends to move in toward the center, leaving the bottom of the outer edge of the cup exposed. When the motion is stopped, the mixture levels out again and covers the bottom of the cup.	800,000 – 5,000,000 cells/ml. 60-70 percent PMN.	Serious Mastitis Check quarters
3	Strong Positive	A gel is formed which causes the surface of the mixture to become Convex. Usually there is a central peak, which remains projecting above the main mass after the motion of the paddle has been stopped. Viscosity is greatly increased so that there is a tendency for the mass to adhere to the bottom of the cup. Mixture becomes slimy or gel like.	Cell number generally over 5,000,000/ml. 70-80 per cent PMN.	Serious Mastitis Check quarters
+	Alkaline Milk pH=7 or over	This symbol should be added to the CMT score whenever the reaction is distinctly alkaline as indicating by contrasting deeper purple color.	An alkaline reaction reflects depression of secretory activity. This may occur either because of inflammation or in drying-off of the gland.	
Y	Acid Milk	Bromcresol purple is distinctly yellow at pH=5.2. This symbol should be added to the CMT score when mixture is yellow.	Distinctly acid milk inside udder is rare. When encountered, it indicates fermentation of lactose by bacterial action within the gland.	

Recovery techniques: Modified authorized food microbiological procedures were verified recovery, isolation, identification and enumeration of *S. aureus* lineage complex from processed mastitic milk (Quinn *et al*., 2004; Jay *et al*., 2005 and BAM, 2020). Mastitic milk from each infected quarter for each examined Cow represent one integrated pooled sample processed separately. Both clinical and subclinical positive CMT processed samples selected for recovery plan. Complementary pooled samples from milk cans in scanned zones in which, Cows were vague. Indirect modified recovery scheme was dependent in which, mastitic milk mixed well and fifteen (15) ml inoculated on sixty (60) ml (modified one part processed sample: five part enriched-enrichment broth) double strengthen powered TSB-YE broth, homogenized well manually, then incubated at 37 °C for 48 hours for resuscitation of suspected sub lethally damaged or stressed isolates. Inoculated propagated samples mixed well again for transferring by standard HiMedia loop about fiver droplets contents (each droplet equal mathematically to 0.02 micron or ml and so on, totally inoculated processed part equal to approximately one ml). Streaking by dilution technique on modified gold standard combo mannitol salt agar pattern A and modified tryptone-soya yeast-extract (TSA-YE) tri molecular Glychophorin blood agars pattern B, then incubated at 37 °C for (24-72) hours followed by cold continuous culturing for blood agars at 4 °C (Hot-Cold haemolysin-siderophores phenomenon).

Enumeration Ecosystem: Authorized systematic culturing on selective and differential modified combo mannitol salt agar plus tri blood agars with verified dual regimes of surface viable micro droplet technique of Miles and Misra (Miles *et al*., 1938) and Pour plate (Soestbergen and Lee, 1969) technique, used for better estimating actual number of *S. aureus* lineage complex in mastitic samples. This modified dual decimal or logarithmic procedure get rid the aerobic and anaerobic environment for missing colonies and so counting errors. The mean log count of recovery of *S. aureus* lineage complex was dependent on colonial phenotypes variants like structures and discoloration phenomenon with haemolytic pattern, colonial biofilm behavior and siderophore activity of isolates. The augmented reality of *S. aureus* lineage complex load log recovery titers calculated via mean number of colonies on cultured plate x a reciprocal of dilution factor x 50 $CFU.ml^{-1}$ (Al-Shammary, 2009).

Segregation-Confirmation Module: Purification and preservation of recovered isolates was proceeds. According to instructions of MacFaddin (2000), Quinn *et al*. (2004) and Microbe online (2019), a Gram stain kit and Capsule staining technique (India ink) used for demonstration of *S. aureus* topographies and insignias with very important feature of present polysaccharide capsule. Formation of faded turbidity on TSB-YE after 48 hours and after 96 hours, a thick and sticky slimy precipitates inside the universals bottoms formed with a characteristic feature of crock-screw motility during agitation, which may indicate a biofilm formation phenomenon in *S. aureus* isolates. This phenomenon also founds in preserved slant seeds after a week. Catalase test preformed to detects the enzymatic activity of *S. aureus* that converts hydrogen peroxide to water and oxygen. An effervescence of oxygen gas within a few seconds indicates a positive reaction. Coagulase test castoff to differentiate *S. aureus* (positive) from Coagulase Negative *Staphylococci* (CONS). Coagulase is an enzyme produced by *S. aureus* that converts soluble plasma fibrinogen into insoluble fibrin triggering agglutination. Virulence biomarker directed toward cell bound tube coagulase dependent index. Coagulation of plasma indicate positive pathogenic strain depending on time of clot formation and degree of agglutination. Highly virulent coagulase negative *S. aureus* variants might encountered. Biochemically, segregation of phenotype variant was a promising feature in clone biotype in which, modified decorum phase buffered system used including characteristic growth pattern on mannitol salt agar with golden pigmentation phenomenon cascaded by Staphyloxanthin pigmentation pattern (dual drift-shift, yellow-white isomers) and epidemiological ancestor on tri molecular phase blood agars with Glychophorin signal.

Biofilm Formation Assay: Formation of biofilm-slim, electromagnetic clouds, charged complex polysaccharide layers matrix pattern needs super nutrient Chemostat power media (extra ATP molecules) to be induced, created and secreted. Qualitative and Quantitavely detection of slime producer strains was determined by culturing the bacteria on modified Tryptone Soy Yeast Extract Broth (TSB-YE) using adherence assay on charged ultra-wide U-shape six-holes tissue culture plates as described previously by Christensen *et al*. (1985). Alternatively, modified Congo red agar (Freeman *et al*., 1989) was dependent for detection of biofilm-producing phenotypes cascaded with the prediction of plasmid comprehending clones phenotypically specially in coagulase negative clones. Creation permission of visible clear biofilm adhesive poly mucoid structures, layers and dots surrounding inside periphery or rims and in the bottom of charged wells indicate positive producers. Optical density (OD) of stained adherent biofilm can be achieved by using micro ELISA auto reader at wavelength ($OD_{570-600}$) nm or real time impedance-based cell analyzer, biosensors, fluorescent- or scanning electron microscopy. Cut-off values for biofilm production can be calculated according to verified methods (Stepanoic *et al*., 2007; Hashem *et al*., 2017; Gutiearrez *et al*., 2016; Larimer *et al*., 2016 and Kirmusaoglu, 2019).

Antibiogram Susceptibility Assay: Proper selection of antibiotic for treatment depends on right diagnostic gesture in which, antibiotics susceptibility test confirm these guidelines. Based on authorized supervisor experience and instructions of national committee of clinical laboratory standards (NCCLS) formerly clinical laboratory standards institute (CLSI (2019) guidelines followed in this account of the Kirby-Bauer disc diffusion method (Bauer, 1966) study the sensitivity of isolates to selected and grouped antibiotics (Bioanalyse® LTD., Turkey) in which, verified test procedure was dependent (Lalitha, 2004 and Quinn *et al*., 2004):

- **Group A:** Penicillin (P 10µ), Ampicillin (AM 10µ), Aztreonam (ATM 30µ), Azithromycin (AZM 15µ) and Amoxicillin / Clavulanic acid (Augmentin, AMC 30µ (20/10).
- **Group B:** Oxacillin (OX 10µ), Cloxacillin (CX 10µ), Methicillin (ME 5µ) and Vancomycin (VA 30µ).
- **Group C:** Ceftriaxone (CRO 30µ), Cephalothin (KF 30µ), Cefixime (CFM 5µ), Cefepime (FEP 30µ), Cefoxitin (FOX 30µ) and Ceftizoxime (ZOX 30µ).
- **Group D:** Imipenem (IPM 10µ), Meropenem (MEM 10µ), Piperacillin (PRL 100µ), Amikacin (AK 30µ), Carbenicillin (PY 100µ) and Bacitracin (B 0.04 u).
- **Group E:** Chloramphenicol (C 30µ), Ciprofloxacin (CIP 5µ), Nitrofurantoin (F 300µ), Trimethoprim / Sulphmethoxazole (SXT 25µ (1.25/23.75), Doxycycline (DO 30µ) and Levofloxacin (LEV 5µ).
- **Group F:** Streptomycin (S 10µ), Optochin (OP 5µ), Clindamycin (DA 2µ), Novobiocin (NV 30µ) and Lincomycin (L 2µ).

ESβL Confirmatory Module: Extended spectrum beta lactamase or Carbapenemases resistance activity was determined in food laboratory for recovered isolates by modified double diffusion inhibition technique or Oxoid Cefpodoxime Combination Kit (Rahma, 2017 and Oxoid-Remel, 2019). Combination discs were a blend of cephalosporin and clavulanic acid on a single disc, which are, used in conjugation with a plain cephalosporin for *in vitro* detection of *ESBLs* strains that do not produce inducible *AmpC* enzymes. The kit contains the following: Cefpodoxime/Clavulanic acid (CD01) 10/1µg and Cefpodoxime (CPD10) 10µg. The presence of Clavulanate enlarged the inhibition zones for all of genetically modified *ESβL*-producing *S. aureus* lineage complex clones by ≥ 5 mm, whereas inhibition zones for Cefpodoxime-susceptible isolates and Cefpodoxime-resistant isolates with *AmpC* and *K1* β-lactamases were enlarged by ≤ 1 mm. Good discrimination was achieved with either the NCCLS (CLSI) or British Society for Antimicrobial Chemotherapy (BSAC Standardized Disc Sensitivity Testing Method) (Carter *et al*., 2000; NCCLS, 2000; Gheldre *et al*., 2003 and Livermore and Brown, 2005). Combination discs should be used by qualified personnel trained to handle category 2 resistant pathogens, and be competent in basic microbiological techniques including antibiotic susceptibility testing. The discs need to be placed on sensitivity media (Muller-Hinton agars) with sufficient space between the discs to allow the formation of clearly defined zones of inhibition and combination of them. A freshly prepared standardized inoculum 0.5 McFarland from each isolate on TSBYE was used in test procedure (4-5 logs equivalent to 10^4-10^5 CFU.ml^{-1}). Zone diameters were measured to the nearest millimeter. A difference of ≥ 5 mm between the zones of the CD01 (10- plus 1-mg) and CPD (10-mg) disks was taken to indicate *ESBL* production, as advocated by the manufacturer. Ceftazidime (CAZ) (30-mg) disks (Oxoid) were tested in parallel as a control discs with kit components in comparison with the susceptibility tables of CLSI (2019) and antibiotics resistance profile index overhead ladders. Alternatively semiconservative protocol was dependent in which, Double-Disc Synergy Test (DDST) elucidate discs containing cephalosporin (cefotaxime or ceftriaxone, ceftazidime, cefepime) were applied next to a disc with clavulanic acid, amoxicillin + clavulanic acid or ticarcillin + clavulanic acid. Positive result was indicated when the inhibition zones around any of the cephalosporin discs were augmented in the direction of the disc containing clavulanic acid. The distance between the discs was critical and 20 mm center-to-center has been found to be optimal for cephalosporin 30 µg discs; however, it may be reduced (15 mm) or expanded (30 mm) for strains with very high or low resistance level, respectively.

Statistical analysis: Bio statistically dependent software of Statistical Package for the Social Sciences (SPSS, version 25 (2019), including t-test and Chi-square for glance significance variations among evoked data.

Results and Discussion

Frequency and distribution pattern of collected and calculated data in Baghdad environment reveled variable interconnected recovery results in phenotypically developed strains of *S. aureus* denominator lineage-complex. Outstanding to quorum sensing biofilm-capsular recalcitrant behavior, multidrug resistance (Combo MRSA-VRSA-ESβL phenotypes), persisters, acids-salts tolerance, thermos-tolerance and genes sharing strategies of these extra plus or professional Gram-positive clustered cocci that synergize actively and passively with other microbiota in clinical cases either like mastitis or inside food environment ecosystem.

Ecomaps segregation chain unveiled recovery of nine isolates out of nintey-pooled samples (10 %) in which, five isolates from Abu-Ghraib (5.56 %), two from Al-Sadrya (2.22 %) and two from Al-Fudhaliyah (2.22 %). Checkerboard resistance pattern unveiled five resistant MRSA-VRSA clones (55.56 %), two ESβL clones (22.22 %) from clinical mastitis in Abu-Ghraib, and one AmpC producers (11.11 %) from subclinical cases in Al-Fudhaliyah with one phenotypes (11.11 %) as intermediate to susceptible. In conclusion, deciphered gadget match of recovery module of biofilm producing and multidrug resistant chimeric strains of *S. aureus* from mastitic milk in Baghdad proved as notifiable emergent new epidemiological cases that needs more sophisticated and verified monitoring programs engaged hazard analysis and critical control points like potential bacteriophages in our food chain ecosystem.

CMT positive traits estimates approximately total leukocytes count predominately PMNs (Leukogram) that predict hygienic status of Cows or pooled raw milk units from territory cans with recovery percentages of *S. aureus* from these samples cross-linked with other contaminants. Results estimated and calculated for clinical and subclinical cases according to selected territories and periods. Tables (2-5) with Figure (1) illustrate these posts.

Table 2 : Grading-Scoring of CMT positive raw milk units replicates from Abu-Ghraib.

Territory	Number of scanned samples	Month	CMT Feedbacks			Grading-Scoring		Predicted-estimated SCCs (PMNs/ml) Cells x log3	
			Positive		Negative				
			Clin	Sub	N	Clin	Sub	Clinical	Subclinical
Abu-Ghraib	30 (5/month)	January	4	3	13	+3	+1	>5,000 70-80 % PMN	400– 1,500 40-60 % PMN
		February	3	3		+2	+1	800– 5,000 60-70 % PMN	400– 1,500 40-60 % PMN
		March	1	2		+1	T	400– 1,500 40-60 % PMN	150– 500 30-40 % PMN
		April	1	0		T	N	150– 500 30-40 % PMN	0
		May	0	0		N	N	0	0
		June	0	0		N	N	0	0
Total		**6**	**17**		**13**				
			30						

Table 3 : Grading-Scoring of CMT positive raw milk units replicates from Al-Fudhaliyah.

Territory	Number of scanned samples	Month	CMT Feedbacks			Grading-Scoring		Predicted-estimated SCCs (PMNs/ml) Cells x log3	
			Positive		Negative				
			Clin	Sub	N	Clin	Sub	Clinical	Subclinical
Al-Fudhaliyah	30 (5/month)	January	1	2	23	+3	+1	>5,000 70-80 % PMN	400– 1,500 40-60 % PMN
		February	1	2		+1	T	400– 1,500 40-60 % PMN	150– 500 30-40 % PMN
		March	0	1		N	T	0	150– 500 30-40 % PMN
		April	0	0		N	N	0	0
		May	0	0		N	N	0	0
		June	0	0		N	N	0	0
Total		**6**	**7**		**23**				
			30						

Table 4 : Grading-Scoring of CMT positive raw milk units replicates from Al-Sadrya.

Territory	Number of scanned samples	Month	CMT Feedbacks			Grading-Scoring		Predicted-estimated SCCs (PMNs/ml) Cells x log3	
			Positive		Negative				
			Clin	Sub	N	Clin	Sub	Clinical	Subclinical
Al-Sadrya	30 (5/month)	January	1	3	18	+3	+1	>5,000 70-80 % PMN	400– 1,500 40-60 % PMN
		February	2	2		+2	+1	400– 1,500 40-60 % PMN	150– 500 30-40 % PMN
		March	1	1		+1	T	400– 1,500 40-60 % PMN	800– 5,000 60-70 % PMN
		April	1	0		N	N	0	0
		May	0	1		N	T	0	800– 5,000 60-70 % PMN
		June	0	0		N	N	0	0
Total		**6**	**12**		**18**				
			30						

Table 5 : Recovery Ecomap of ***S. aureus*** lineage complex from CMT posted raw milk units (Territory).

Territory	Brands NO.	CMT Feedbacks Responses			Recovered ***S. aureus*** clones			Recovery Ecomap %		
		Clinical	**Subclimax**	**Normal**	**Clinical**	**Subclimax**	**Normal**	**30**	**90**	**450**
Abu-Ghraib	30	9 ^{Aa}	8 ^{Aa}	13 ^{Ab}	**4** ^{Ab*}	1 ^{Ac}	7 ^{Aa}	40	13.33	2.67
Al-Fudhaliyah	30	2 ^{Ca}	5 ^{Bb}	23 ^{Cc}	1 ^{Ba}	1 ^{Aa}	1 ^{Ca}	10	3.33	0.67
Al-Sadrya	30	5 ^{Ba}	7 ^{Aa}	18 ^{Bb}	1 ^{Bb}	1 ^{Ab}	4 ^{Ba}	20	6.67	1.33
Total	90	16	20	54	6	3	12	70	23.33	**4.67**

CODEBOOK		
	Territories	**A,B,C:** Bio statistically indicate significant differences **vertically** among **territories** at level **(P≤0.05).**
		a,b,c: Bio statistically indicate significant differences **horizontally** within **territory** at level **(P≤0.05).**
	Isolates	**A,B,C:** Bio statistically indicate significant differences **vertically** among **isolates** at level **(P≤0.05).**
		a,b,c: Bio statistically indicate significant differences **horizontally** within **isolates** at level **(P≤0.05).**
		:** Indicate highest isolation ratio of clinically ***S. aureus lineage complex from **Abu-Ghraib** territory.

Table 6 : Recovery Ecomap of *S. aureus* lineage complex from CMT posted raw milk units (Period).

Periods	Brands NO.	CMT Feedbacks Responses			Recovered ***S. aureus*** clones			Recovery Ecomap %		
		Clinical	**Subclimax**	**Normal**	**Clinical**	**Subclimax**	**Normal**	**15**	**90**	**450**
January	15	6 ^{Aa}	8 ^{Aa}	1 ^{Ab}	**3** ^{Ab*}	1 ^{Ac}	5 ^{Aa}	73.33	12.22	2.44
February	15	6 ^{Aa}	7 ^{Aa}	2 ^{Ab}	1 ^{Ba}	1 ^{Aa}	2 ^{Ba}	20	3.33	0.67
March	15	2 ^{Ba}	4 ^{Bb}	9 ^{Bc}	1 ^{Bb}	1 ^{Ab}	2 ^{Bb}	40	6.67	1.33
April	15	2 ^{Bb}	0 ^{Dc}	13 ^{Ca}	1 ^{Ba}	0 ^{Cb}	1 ^{Ba}	6.67	1.11	0.22
May	15	0 ^{Cc}	1 ^{Cb}	14 ^{Ca}	0 ^{Cb}	0 ^{Cb}	1 ^{Ba}	0	0	0
June	15	0 ^{Cb}	0 ^{Db}	15 ^{Ca}	0 ^{Cb}	0 ^{Cb}	1 ^{Ba}	0	0	0
Total	90	16	20	54	6	3	12	140	23.33	**4.67**

CODEBOOK		
	Periods	**A,B,C,D:** Bio statistically indicate significant differences **vertically** among **periods** at level **(P≤0.05).**
		a,b,c: Bio statistically indicate significant differences **horizontally** within **period** at level **(P≤0.05).**
	Isolates	**A,B,C:** Bio statistically indicate significant differences **vertically** among **isolates** at level **(P≤0.05).**
		a,b: Bio statistically indicate significant differences **horizontally** within **isolates** at level **(P≤0.05).**
		:** Indicate highest isolation ratio of clinically ***S. aureus lineage complex throughout **January** period.

Fig. 1 : Deciphered CMT positive checkpoints box (10 MPX).

Clinically and bio statistically ecomaps segregation chain deciphered premier and significant isolation ratio or recovery log decorum was from Abu-Ghraib territory particularly from normal raw milk units replicates and from clinical versus subclinical cases of mastitis in comparison to Al-Sadrya territory cascaded by Al-Fudhaliyah territory respectively. All recovered isolates clones showed versatile growth curve and pattern cascaded by generation time denominator (deciphered phenotypic cascaded by genotypic drift and shift interconnected isomers). Frequency and distribution pattern of deciphered *S. aureus* ecotope from mastitis cases especially subclinical forms represent major and serious problem for both man and animals versus ascending-descending, contamination-pollution ecosystem with normally recovered phenotypes. Variable controversial, adapted and changed data behaviors reveled. Polymorphic colonial forms remarked. Small colonial variants (SCVs), medium, large, mega biofilm to fungus crenated transformed to Medusa entity phenotypes demonstrated on combo modified mannitol salt agar pattern-A plus modified TSA-YE tri molecular blood agars pattern-B. Pathogenicity profile index cascaded by virulence pigmentation-discoloration revel segregation pattern of *S. aureus* colonies into dual phase as whitish and yellowish Staphyloxanthin behaviors on modified blood agars qualitatively from clinical and subclinical cases of mastitis versus normal raw milk units posted by CMT module. False positive and false negative receiver operating characteristics (ROC values) for these phenomenal behaviors would complete the hidden features of recovered deciphered entities.

Most SCVs variants were non-biofilm white to yellow while; most large phenotypes were yellowish to golden biofilm structures during precise checking of plates with loop or ghostly configurations inside TSB-YE broths. The other side of trueness reveled versatile development and transformation of SCVs phenotypes from non-biofilm entity to a Medusa head creature. Different haemolytic features noticed with biofilm texture. Complete clear alpha concomitant with incomplete beta hue green haemolytic pattern, and even gamma non-haemolytic siderophore activity found here. Close-Up feature was predominant in Al-Fudhaliyah territory. Close-Up and chain or beads colonial behaviors growth patterns on blood agars concomitant with biofilm-encapsulated features of intestinal-like folds, stars, teeth and even horseshoe patterns noticed also. Direct clues or observed arrays inshore presence of final stage of complex extra biofilm pregnant-like creatures of enclosed-folded vesicles or bellies of Medusa entity loaded with new generations of *S. aureus* offspring from forbidden zones of Devils Medusa head colonies. Variable counting logs among polymorphic phenotypes of *S. aureus* lineage complex might indicate primary and secondary interconnected infectious cycle ascending or descending (environmental versus contagious) with predisposing factors like climatic and environmental niches. Recovery Tables (7 and 8) and Figure (2) segregated denominator ecomap concussions according to infected territory and periodic tables from January to June.

Table 7 : Recovery mean log count of *S. aureus* lineage complex (CFU.ml^{-1}) from clinical mastitis.

Territory	No. Brands	*S. aureus* (CFU.ml^{-1}) Plethora		Total Log
		SCVs phenotypes	*Large* phenotypes	
Abu-Ghraib	30	4.477 ^{Ba}	3.837 ^{Ab}	8.314 ^{A}
Al-Fudhaliyah	30	None ^{Ca}	2.588 ^{Bb}	2.588 ^{C}
Al-Sadrya	30	3.342 ^{Aa}	None ^{Cb}	3.342 ^{B}
Total	90	2.606 ^{a}	2.141 ^{b}	4.748

A,B,C: Indicate bio statistically significant differences **vertically** among **territories** at level (**P≤0.05**).
a,b: Indicate bio statistically significant & clinical differences **horizontally** within **colonial variants** at level (**0.5> count >0.5 log**).

Table 8 : Recovery mean log count of *S. aureus* lineage complex (CFU.ml^{-1}) from subclinical mastitis.

Territory	No. Brands	*S. aureus* (CFU.ml^{-1}) Plethora		Total Log
		SCVs phenotypes	*Large* phenotypes	
Abu-Ghraib	30	5.005 ^{Aa}	6.380 ^{Ab}	11.385 ^{A}
Al-Fudhaliyah	30	None ^{Ba}	5.021 ^{Bb}	5.021 ^{C}
Al-Sadrya	30	4.653 ^{Aa}	4.301 ^{Ca}	8.954 ^{B}
Total	90	3.219 ^{a}	5.234 ^{b}	8.453

A,B,C: Indicate bio statistically significant differences **vertically** among **territories** at level (**P≤0.05**).
a,b: Indicate bio statistically significant & clinical differences **horizontally** within **colonial variants** at level (**0.5> count >0.5 log**).

CHECKPOINTS BOX 1: From cross-tables overhead estimated and calculated data reveled bio statistically significant differences vertically among territories at level (P≤0.05), with encountered clinical differences horizontally within colonial variants at level (0.5> count >0.5 log). According to standardized microbial log count formula of Jay (2005) in food microbiology in which, each decreasing or increasing 0.5 log count of microbe in food indicate clinically significant differences affecting hygienic status and this might not in necessary indicate bio statistical Interface marginal true differences. In some cases especially recovery of one log count or one *S. aureus* (CFU.ml^{-1} or g food) of extra plus pathogenic environmental versus contagious entity indicate significant risk on animals and their products (food chain) ending with serious hazard sequel on human hygiene (Coles, 1987). All so, total log counts in both clinical and subclinical mastitis exceeding 6 logs (10^6) CFU.ml^{-1}, this exaggerate the risk problems especially in subclinical cases with these doubled shield recalcitrant foci of encapsulated and biofilm producing clones of *S. aureus* matrixed with other contaminants in same samples units particularly focused in Abu-Ghraib territory. The question here, which clone or mixed clones was or were the primary or secondary cause of infection in presence of complicated environmental versus contagious ecomaps' segregation chain affecting extended ecosystem of lactating Cows and milk cans in selected involved territories where samples engaged. None SCVs phenotypes in Al-Fudhaliyah territory versus none Large phenotypes in Al-Sadrya territory was observed, this might be indicate clever and modified behavioral deciphered strategies in organization and redirection of their sophisticated hidden repairing quorum and vice versa their homeostasis's. Besides encountered predictive CMT data, the other side of trueness, says that not all increased leukogram's count over five logs of PMNs ml^{-1} of raw milk indicate a mastitis (false positive). Different scenarios discussed such as species variations, stage of lactation, age of animal, traumatic injuries, descending disease and so on. Versus not all decreasing in leukogram's count below five log indicate normal raw milk or absence of mastitis (false negative) like hidden early stages of subclinical mastitis that cannot be detected precisely with predictive tests like CMT alone. Therefore, CMT is a predictive screening test depends on both microbial and leukocytes counts (Dual phase: Total and differential), that must be completed with other specificity and sensitivity recall tests such as *in vitro* nitro blue tetrazolium reduction assay and *in vivo* carbon clearance assay or other immune qualitative assays that check phagocytic activity of PMNs to inshore segregation of normal raw milk from mastitic once.

Fig. 2 : Deciphered combo recovery of *S. aureus* lineage complex from CMT units on modified mannitol salt and blood agars with dual white-yellow pattern (10 MPX).

Polydenomorphism phenomenon unveiled during achievement of individual variation of genetically modified denominator *S. aureus* clones. Due to quorum sensing mechanisms and genes sharing strategy module during symposium ecosystem with ubiquitous Eco biota inside different environmental niches like intestine, mastitis and during synthesis of dairy products. With the development of hidden and sophisticated strategies to production and accusation of iron chelating glycoproteins' like siderophores. Data reveled the ability of different recovered clones from all samples to lysis of different types of RBCs with variable zones of haemolysis (diameter in millimeter) depending on the power of isolate, genetic nature, their haemolysins and siderophores, pathogenicity index for irons (ferric and ferrous) and regulatory bionetworks. Different haemolysins pattern unveiled in *S. aureus* axis from incomplete beta dark-hazy green hues under the colonies to complete alpha isomers with clear zone of haemolysis surrounding the colonies with different zones diameters depending on the requests of colony forming unit for irons i.e. degree of virulence and types of siderophores gamma haemolysins present in areas of growth on blood agars.

Highly virulent clones' requests low triggering irons versus low virulent clones requests high levels of iron but both requests high levels during proceedings cycles of pathogenicity inside host. These pathogenic features of haemolysins and siderophores noticed not only from clinical and subclinical cases of mastitis but also from clones distributed and recovered from yogurt and soft cheese-whey ecosystem. This might be indicate strong and interconnected relationship among human, animals and environmental isolates in association with other microbiota in samples collected and examined. Hot-Cold phenomenon noticed here after incubation for another 24 hours inside the refrigerator, due to organized activity lifestyle homeostasis. Siderophore profile represent here diameter in millimeter of zones of haemolysis for alpha type in which, it is a linked glycoprotein with haemolysin either as a different moiety or as gamma type, and diffusible glycoprotein. Epidemiological segregation of isolates according to type of haemolysis into HUMAN-specific (mostly nosocomial) lyse only human RBCs, LIVESTOCK isotype lyse only Sheep and Ox RBCs, ZOONOTIC-REVERSE ZOONOTIC profile (mostly communicable) lyse both RBCs and ENVIRONMENTAL phenotype not lyse any type of RBCs but might contain invisible functional isomer of gamma type haemolysin-siderophore glycoprotein. Staphyloxanthin pattern was variable in versatile recovered colonies according to their epidemiological genetic makeup, versatile size polymorphism, haemolysins-siderophore index and case history of sample. These features were posted in tables (9-11) and Figure (3).

Table 9 : Hemolysis Pattern, Siderophore Profile and Staphyloxanthin Index of clones recovered from MASTITIS on modified Human TSAYE-Blood agars after (24-72) hours at 37 and 4 °C.

TERRITORY	Abu-Ghraib (5)				Al-Fudhaliyah (2)				Al-Sadrya (2)			
Codebook Case	Clinical (4)		Sub (1)		Clinical (1)		Sub (1)		Clinical (1)		Sub (1)	
Colony polymorphism	L	SCV	L	SCV	L		L		L	SCV	SCV	
Haemolysis Pattern	α	α	α	α	α		α		α	β	β	
	β	β	β	β	β		β					
Siderophore profile	10 mm		(5-10) mm		(2-8) mm		2 mm		(3-10) mm		10 mm	
Staphyloxanthin Index	+ ve (3)	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve	+ ve	-ve	+ ve	-ve

Table 10 : Hemolysis Pattern, Siderophore Profile and Staphyloxanthin Index of clones recovered from MASTITIS on modified Sheep TSAYE-Blood agars after (24-72) hours at 37 and 4 °C.

TERRITORY	Abu-Ghraib				Al-Fudhaliyah				Al-Sadrya			
Codebook Case	Clinical (4)		Sub (1)		Clinical (1)		Sub (1)		Clinical (1)		Sub (1)	
Colony polymorphism	L	SCV	L	SCV	None				None			
Haemolysis Pattern	α											
Siderophore profile	5 mm											
Staphyloxanthin Index	+ ve (3)	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve	+ ve	-ve	+ ve	-ve

Table 11: Hemolysis Pattern, Siderophore Profile and Staphyloxanthin Index of clones recovered from MASTITIS on modified OX TSAYE-Blood agars after (24-72) hours at 37 and 4 °C.

TERRITORY	Abu-Ghraib				Al-Fudhaliyah				Al-Sadrya			
Codebook Case	Clinical (4)		Sub (1)		Clinical (1)		Sub (1)		Clinical (1)		Sub (1)	
Colony polymorphism	L	SCV	L	SCV	L		L		L	SCV	SCV	
Haemolysis Pattern	α				None		α		None			
Siderophore profile	(5-15) mm						10 mm					
Staphyloxanthin Index	+ ve (3)	-ve	+ ve	-ve	+ ve	+ ve	+ ve	+ ve	+ ve	-ve	+ ve	-ve
Epidemiological Pattern	**Zoonotic Reverse Zoonotic**				**Human**		**Zoonotic**		**Human**			

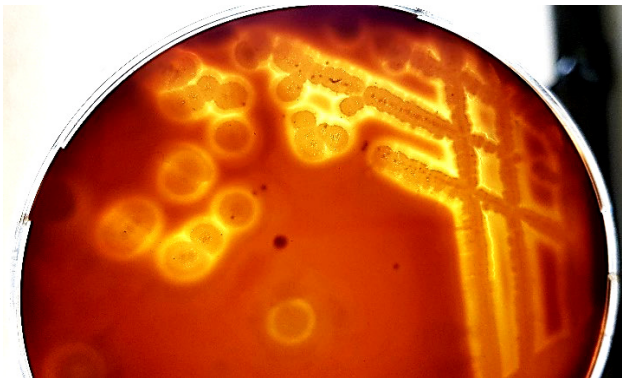

Fig. 3 : Haemolysis pattern, Siderophore profile and Staphyloxanthin pigmentation of *S. aureus* on modified blood agar.

Photographic data reveled blue and red adhesive circles of multilayered slim surrounding the interior rims of tissue culture microtiter plate holes as well as discolored scattered dots on the bottom of holes. Sensitivity and specificity of modified dual regimes of crystal violet-safranin technique revised by Supervisor Al-Shammary could discriminate between clones and within phenotypes in virulence degree as an index of plasmid and extended-spectrum for resistance of antibiotics with indirect arrays of foodborne modified CRISPR-CAS clones of *S. aureus*. Figure (4) below revised these featured behaviors.

Fig. 4 : Biofilm architecture geometry and matrix entity.

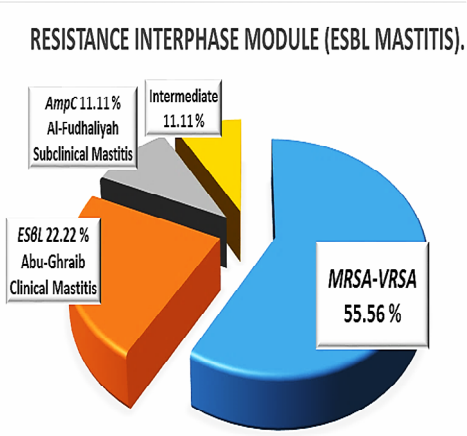

Fig. 5 : Resistance and Susceptibility interphase module of recovered MASTITIS isolates to selected group (A, B, C, D, E, F and ESβL) of antibiotics according to CLSI and BSCA (2019-2020) Codebook.

Correct and accurate selection of antibiotic for treatment depends on right diagnostic gesture in which, antibiotics susceptibility test confirm these guidelines. Combo segregation of recovered clones and their versatile phenotypes into resistant, tolerant, persisters, intermediate and susceptible entities depends on ecosystem of samples and territories guided by their broad-spectrum genetic module (interconnected drift and shift module among recovered clones and even within their phenotypes). Combo deciphered and verified Kirby-Bauer disc diffusion technique combined with Carbapenems Cefpodoxime Combination Kit and Ceftazidime (CAZ) susceptibility module assist in segregation-confirmation scheme. Otherwise, Vitek-2 dependent bionetwork clearly distinguish between resistance series and epidemiological biotypes. Depending on guidelines standardized criteria of based tables of antibiotics susceptibility zones with MIC and MBC threshold breakpoints of CLSI and BSCA for *S. aureus* combined followed by interconnected merged zones of Cefpodoxime/ clavulanic acid (CD01) with Cefpodoxime (CPD10) controlled by Ceftazidime (CAZ) susceptibility module in which, a difference of ≥ 5 mm between the zones of the CD01 (10- plus 1-mg) and CPD (10-mg) disks was taken to indicate ESβL producing strains that do not produce inducible AmpC enzymes., as advocated by the manufacturer. Selected antibiotics here reflect flexibility and complexity of the test procedure in which, we select here thirty-five types of different ordinary to new generations of antibiotics used in veterinary and human medicine. The legacy, integrity, concentration and grouping of them listed in reference table's vs dependent Standard coding in order to compare and judgment of primed and calculated zones of inhibitions (diameter in millimeter) for each antibiotics for each selected clone. Interconnected Codebook Figure (5 and 6) bellow reflect these tested behaviors.

CHECKPOINTS BOX 2: Mastitis ecomaps segregation chain deciphered recovered clones from detailed upstairs flowcharts of antibiotics resistance cascaded by ESβL combination discs and CAZ interphase module into versatile entities. Abu-Ghraib clones displayed variable interconnected degrees of susceptibility pattern in comparison to other resistant phenotypes recovered from Al-Fudhaliyah and Al-Sadrya. Some phenotypes from Abu-Ghraib displayed fluctuated degrees of resistance bridges from intermediate to sensitive bridge cascaded by intermediate to Resistance bridge to Azithromycin (AZM 15µ), Chloramphenicol (C 30µ), Ciprofloxacin (CIP 5µ), Levofloxacin (LEV 5µ), Streptomycin (S 10µ), Clindamycin (DA 2µ), Novobiocin (NV 30µ) and Lincomycin (L 2µ). Most versatile clones among territories and within their large and SCVs phenotypes displayed tolerant-persisters (natural or mutant) colonial variants as discrete entities inside inhibition zones. Overall total resistance percentages module in this sector disintegrated into five resistant MRSA-VRSA clones (55.56 %), two ESβL clones (22.22 %) from clinical mastitis in Abu-Ghraib, and one AmpC producers (11.11 %) from subclinical cases in Al-Fudhaliyah with one phenotypes (11.11 %) as intermediate to susceptible. These valuable criteria when merged with upstairs behaviors of haemolysins, siderophores and Staphyloxanthin pigmentation (diffuse yellowish, brownish and greenish octopus like ink) cascaded by recalcitrant capsule-biofilm electromagnetic shield and catalase-oxidase-coagulase-gelatinase messengers leading us to putting predicted and calculated results as ROC curve or Medusa Checkerboard.

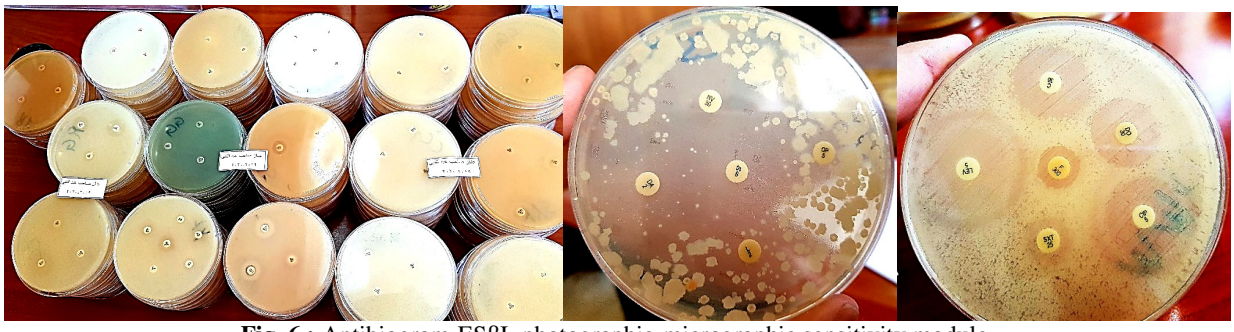

Fig. 6 : Antibiogram-ESβL photographic-micrographic sensitivity module.

Deciphering depending on the authorized experience and visions guidelines of supervisor researches on multidrug resistant foodborne microorganisms and on linked or associated previous researchers on methicillin and vancomycin resistant clones of *S. aureus* lineage-complex (Kanaan, 2013; Kanaan and Al-Shammary, 2013; Al-Shammary, 2009; Al-Shammary and Abdul Mounam, 2011; Al-Shammary, 2015a and 2015b; Al-Shammary *et al*., 2015; Al-Shammary and Madi, 2016; Al-Shammary, 2017; Al-Shammary and Abdul Mounam, 2017; Al-Shammary, 2019 and Alyais, 2019). Upgraded electromagnetic bridges of biofilm entity in these developed and genetically well-equipped chimeras reflect their potential hazard policy for man, animals, food, water and environment. Uncontrolled importation of contaminated feeds-foods recycled contaminated Iraqi environment with foreign clones carrying strong and intelligent defense barriers strategies called CRISPR-CAS immune system cascaded and regulated cleverly by quorum after 2003. All these scenarios with other obscure causes leading to emergency of these resistant and recalcitrant persisters biofilm clones. Acquisition and accumulation of inserted foreign genetic material rather than mutation in Enterotoxigenic *S. aureus* Iraqi clones due to symposium relationship among and within this Eco biota in the same niche ecosystem through years leading to sophisticated genes sharing strategies either by conjugation plasmids bridges or by transduction with pathogenic prophage or throughout transformation by residual forbidden genetic material (environmental or eDNA) cascaded by abnormalities in intentional redirected genetic engineering and programed penicillin binding proteins of *mecA* genes of staphylococcal pathogenicity islands versus staphylococcal chromosome cassette islands regulated by di headed chaperone accessory gene regulator and Sigma factors linked with acquisition of foreign sets of antibiotics resistance represented by extended-spectrum beta lactamase and Carbapenemase from Gram negative pathogens versus interphases positively-negatively charged zwitterion capsule ending with transformation to armamentarium entity of Medusa. Adaptation of *S. aureus* lineage-complex to broad-spectrum hosts and food ecosystems with the generation of diverse and versatile antigenic drift and shift in new redesigned clones with new emergent and hazardous capacity to remodeling their homeostasis with stress hardening and ballooning theory to overcome or resist different encountered environmental harsh modules (Rowan and Anderson, 1998; Al-Shammary, 2009; Xia and Wolz, 2014). Understanding clever switching between drift and shift behavior under accessory gene regulator quorum sensing mechanisms could illustrate some struggling *in vivo*, *in vitro* and even *in situ* of these recovered creatures.

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