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# Effect of Some Disinfectants on Antibiotic Resistance Staphylococcus Isolated from Dairy Farms in Egypt.

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***Abstract:*** *Antibiotic resistant staphylococci are major public health concern since the bacteria can be easily circulated in the environment rather than coagulase negative staphylococcus can be involved in mastitis infection, resulting on reduced production of milk and decreased quality, causing the most important economic losses in the dairy industry.*
***Objective:*** *is studying the effect of some commercially available disinfectants on four species of antibiotic resistant staphylococci (Staphylococcus intermedius,Staphylococcus xylosus, Staphylococcus hyicus and Staphylococcus sciuri) isolated from dairy farms in Egypt.*
***Methods:*** *Bactericidal potency was measured by the logarithmic reduction factors (LRFs) achieved with each strain using suspension tests PrEN 1276(2009). Surface test was performed using* ***PrEN 13697(2001)***
***Results:*** *Zixvirox [R] 0.5% achieved the required logarithmic reduction >5 after one-minute contact time followed by Virkon S 1%while Dyne-O-Might[R]1% and POLYCAR[R] 3% were not achieved the required logarithmic reduction and not considered bactericidal agent. The results of surface test using the same strains and disinfectants after five minutes contact time were somewhat lower than that obtained by suspension test.*
***Conclusion:*** *although all strains are staphylococcus antibiotics resistant strains they differ in their responses to different bactericidal agents.*
***Key words****: Staphylococcus, antibiotic resistant, disinfectants, bactericidal, Carrier test, Dairy industry.*

## I. Introduction

Antibiotic resistant staphylococci are major public health concern since the bacteria can be easily circulated in the environment and infections due to these strains could be difficult to be treated [1]**.**

Mastitis is one of the most important problems for ruminant dairy herds around the world. Economic losses are associated with the drop in quantity and quality of milk production, as well as the increased costs involved in treatment and control programs [2].

Recent studies have indicated that most cases of clinical mastitis occurring in dairy cows in developed dairy regions are caused by environmental pathogens [3].

Coagulase-negative staphylococci (CNS), have become the most commonly isolated microorganisms from bovine milk in many countries and are regarded as emerging mastitis pathogens [4] and resulting on reduced production of milk and decreased quality, causing the most important economic losses in the dairy industry [5]. In addition, CNS are abundantly present both in the cows' environment [6] and on their teat apices [7]. Also CNS showed high virulence by their ability to form biofilm which also get cells in biofilm more resist to antimicrobial agent [8]. Staphylococcus strains with mecA are resistant to lactam antibiotics and frequently code for multi-drug resistance, which may represent a serious health and economic concern [9]. Consequently, it is highly important to detect mecA, especially in all Staphylococcus strains [8].

The resistance genes might in some instances transfer from staphylococci of animal origin to staphylococci that cause infections in humans, thereby compromising antimicrobial treatment [10]. CNS colonizing the udder of buffaloes and cows may represent a reservoir of different antibiotic resistance genes and Staphylococcal Cassette Chromosome mec (SCCmec) elements. Could this genetic background could be a reservoir for interspecies gene transfer among CNS and S. aureus in the udder as it was previously suggested in the intestinal tract [11].

Therefore, it is highly important to detect mecA, especially in Staphylococcus samples. In recent years, increasing numbers of reports have shown that the mecA gene is present in CNS strains, including hospital-acquired infections, neighborhoods [12]. Determining environmental and human Staphylococcus reservoirs and providing proper hygiene and proper disinfection methods was needed to control infection [13].This work was carried out to study the effect of four available disinfectants on four strains of antibiotic resistant staphylococci isolated from dairy farms in Egypt and the bactericidal potency was measured by the logarithmic reduction factors (LRFs) achieved with each strain using suspension test and surface test.

## II. Materials:

### 2.1 Chemical disinfectants:

Four chemical disinfectants were tested individually for 1 and 5minutes contact time. The used disinfectants were:

**Table (1) Active component & Manufactures of Bactericidal Agent**

| | ACTIVE CHEMICAL | MANUFACTURE |
|---|---|---|
| DYNE-O-MIGHT$^{R}$ 1% | IODINE 0.42% AND ORGANIC ACID | PRESERVES INTERNATIONAL 66171, RENO , NEVADA, USA 89511 |
| ZIXVIROX $^{R}$ 0.5% | HYDROGEN PEROXIDES 25% AND PERACETIC ACID 5% | BBZIX COMPAY, SPAIN |
| POLYCAR $^{R}$ 3% | SODIUMALKYLSULFATE 3.4 %, ALKYLARYLPOLYGLYCOLETHERSULFATE 4.5%, FATTYALCOHOLEETHOXYLATE 4.4%,BUTYLGLYCOLE 4.5%,TETRAPOTASSIUMPYROPHOSPHATE 5.0%, SODIUMTRIPOLYPHOSPHATE 2.5%,CAUSTIC SODA 1.0%.. | EWABO COMPANY:, GERMAN |
| VIRKON S $^{R}$1% | POTASSIUM PEROXYMONOSULFATE 21.4% , SODIUM CHLORIDE 1.50% AND OTHER INGREDIENTS | ANTEC INTERNATIONAL LTD,SUDBURY,SUFFOLK,ENGLAND |

### 2.2 Interfering agent:

#### 2.2.1 Sterile hard water

The tested disinfectants were diluted using **400** ppm hard water solution on the day of use. The hard water solution was as following:

1. 972 ml bi-distilled water
2.12ml solution A (19.84 g anhydrous MgCl2)+ (46.24 anhydrous CaCl2)/ L
3. 16ml solution B (35.02 g NaHCO3/L)

#### 2.2.2 Organic matter:

5% yeast extract powder solution was prepared by adding 5g yeast extract to 100ml bi- distilled water as a source of organic matter.

### 2.3 Neutralizer.

**Table (2) Composition of Neutralizing Agent**

| DISINFECTANT AGENT | NEUTRALIZER | REFERENCE |
|---|---|---|
| FOR ALL THE DISINFECTANTS | A COMBINATION OF 3% TWEEN 80 (POLYSORBATE 80), 0.3% LECITHIN, 0.1% HISTIDINE, 0.5% SODIUMTHIOSULPHATE, 3% SAPONIN AND 1% SODIUM LAURETH SULPHATE. | ASTM E 1054-02 [14]**.** |

### 2.4 Bacterial strain and growth conditions.

The four isolates were identified as CNS based on Coagulase test using both the slide and tube methods. Coagulase-negative isolates were subjected to identification to the species level using the API Staph commercial identification system (API Staph ID32 test; bioMérieux, Marcyl' Etoile, France) [15]. Also the antibiotic resistance of the four strains were determined phenotypically using disk diffusion test and resistance was determined by measurement of inhibition of growth around the antimicrobial disk according to the zone diameter interpretative standards of CLSI [16] according to the antimicrobials manufacturers' instructions and genotypically using duplex PCRfor amplification of blaZ gene (a determinant of β- lactamase production) according to Vesterholm-Nielsen et al. [17] and mecA gene (a determinant of methicillin resistance) according to Zhang et al. [18]**.**

**Table 3. Biochemical& Genotypic resistance genes**

| ISOLATES | PHENOTYPIC EXAMINATION | | | | GENOTYPIC DETECTION OF ANTIBIOTIC RESISTANCE GENE | |
|---|---|---|---|---|---|---|
| | CATALASE TEST | OXIDASE TEST | OXIDATIVE FERMENTATION TEST | COAGULASE TEST | BLAZ GENE | MECA GENE |
| **S.INTERMEDIUS** | +VE | -VE | F | -VE | +VE | +VE |
| **S.XYLOSUS** | +VE | -VE | F | -VE | +VE | +VE |
| **S.SCIURI** | +VE | -VE | F | -VE | +VE | +VE |
| **S.HYICUS** | +VE | -VE | F | -VE | +VE | +VE |

**Table 4 - Antibiogramme ofStaphylococcus**

| ISOLATES | DISK DIFFUSION TEST | | |
|---|---|---|---|
| | PENICILLIN | AMOXICILLIN | OXACILLIN ACID |
| **S.INTERMEDIUS** | R | R | R |
| **S.XYLOSUS** | R | R | R |
| **S.SCIURI** | R | R | R |
| **S.HYICUS** | R | R | R |

The isolates were maintained in pure culture on nutrient agar slants. Cultures were streaked for isolation on mannitol salt agar and incubated for 24 h at 37°C. Then cells were suspended in peptone physiological salt solution (PPS) (1 g of neutralized bacteriological peptone per liter, 8.5 g of NaCl per liter) to an optical density at 620 nm corresponding to a concentration of $1.5 \times 10^8$ to $5 \times 10^8$ CFU ml$^{-}$

## III. Methods

**3.1** Evaluation of disinfectants is often based on laboratory suspension tests according to PrEN1276 [19]. 1 mL of a bacterial test suspension adjusted to $1.5 \times 10^8$ to $5.0 \times 10^8$ cfu/mL using McFarland standard and was added to 1 mL interfering substance. (5% yeast extract). The mixture was maintained at 20°C±1°C for 2 min ±10 s. Then 8 mL of the product test solution were added and the mixture was maintained at 20°C±1°C for 1, and 5 min exposure time. At the end of the contact times an aliquot was taken and the bactericidal activity in this portion was immediately neutralized or suppressed by dilution-neutralization method adding 1 mL sample to a tube containing 8 mL of specific neutralizer dissolved in Tryptone Soya Broth 30.0 g/L and 1 mL water mixed by vortexing, and left at 20oC. After 5 min neutralization time, duplicate 1.0ml volumes were pour plated with tryptone soya agar and incubated at 37oC for 48 hr prior to counting. The microbicidal effect (ME) was calculated by subtracting the log of viable count after disinfection (Na) from the log of the initial count in the bacterial test suspension (N x 10-1). The products must achieve a five log reduction in viable counts (ME value of 5 or higher) to accept as a microbicidal compound.

**3.2 Surface test:**

The surface test based on the surface test method described in PrEN 13697 [20] which specifies a quantitative surface test for the evaluation of bactericidal activity of chemical disinfectants used in the food industry ,stainless steel Coupons measuring 1.5 x 2 cm were autoclaved at 121°C for 15 min, To prepare the test suspension two minutes prior to the actual test 1 mL of the bacterial test suspension containing $1.5 \times 10^9$ to $5.0 \times 10^9$ cfu/mL was added to 1 mL of the interfering substance(yeast extract 5%) and mixed. The test surfaces were placed in an open Petri dish ensuring that the stainless steel coupons were in horizontal position. Then they were inoculated with 0.05 mL of the test suspension and interfering substance mixture and dried in an incubator at 37°C for 45-55 min until they were visibly dry. After drying the temperature of the surface was adjusted to room temperature. Then the inoculum was covered with 0.1 mL of the product test solution, or for the water control with water of standardized hardness instead of the product. After the chosen exposure times of 5 min the surfaces were transferred into separate flasks containing 10 mL of an appropriate neutralizer and glass beads.

After a neutralization time of 5 min a series of tenfold dilution were prepared in Tryptone-NaCl solution. The number of surviving test organisms was determined quantitatively. For each test organism, product test concentration and exposure time, the reduction in viability in comparison to the water control was calculated.

## IV. Results And Discussion

**Table (5)** Staphylococcus log reduction on suspension test after one minute:

| | **DYNE-O-MIGHT 1%** | **ZIXVIROX 0.5%.** | **POLYCARP 3%** | **VIRKON S 1%** |
|---|---|---|---|---|
| S. INTERMEDIUS | 1.5 | 6.8 | 2.1 | 7.1 |
| S.XYLOSUS | 1.5 | 6.1 | 2.0 | 3.0 |
| S.HYICUS | 3.1 | 7.1 | 1.4 | 3.0 |
| S.SCIURI | 3.3 | 6.2 | 1.9 | 4.0 |

**Fig( 1)** Staphylococcus log reduction on suspension test after one minute contact:

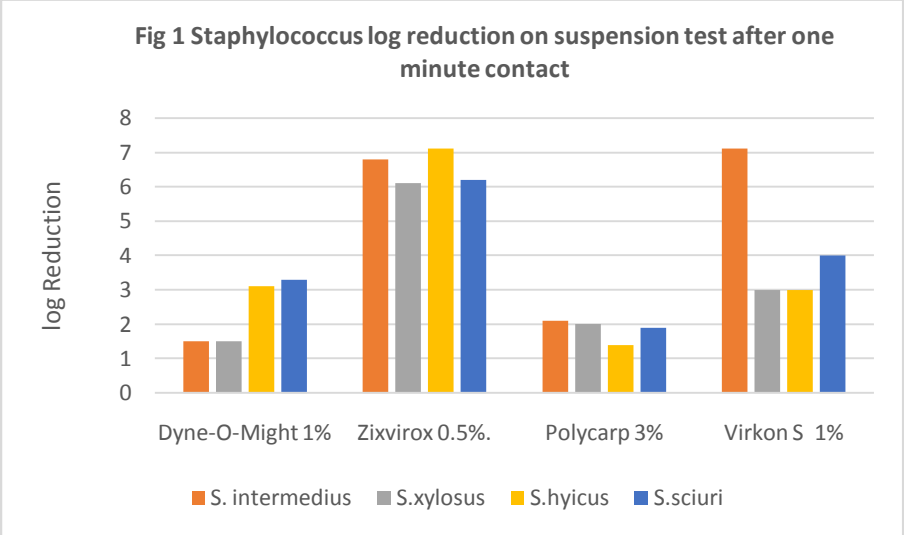



**Table (6)** Staphylococcus log reduction on suspension test after five minutes contact:

| | DYNE-O-MIGHT 1% | ZIXVIROX 0.5%. | POLYCARP 3% | VIRKON S 1% |
|---|---|---|---|---|
| S. INTERMEDIUS | 3.1 | 8.1 | 2.8 | 8.1 |
| S.XYLOSUS | 4.2 | 7.1 | 2.8 | 5.3 |
| S.HYICUS | 6.5 | 8.1 | 3.1 | 5.2 |
| S.SCIURI | 3.7 | 6.5 | 3.4 | 6.1 |

**Fig. (2)** Staphylococcus log reduction on suspension test after five minutes contact:

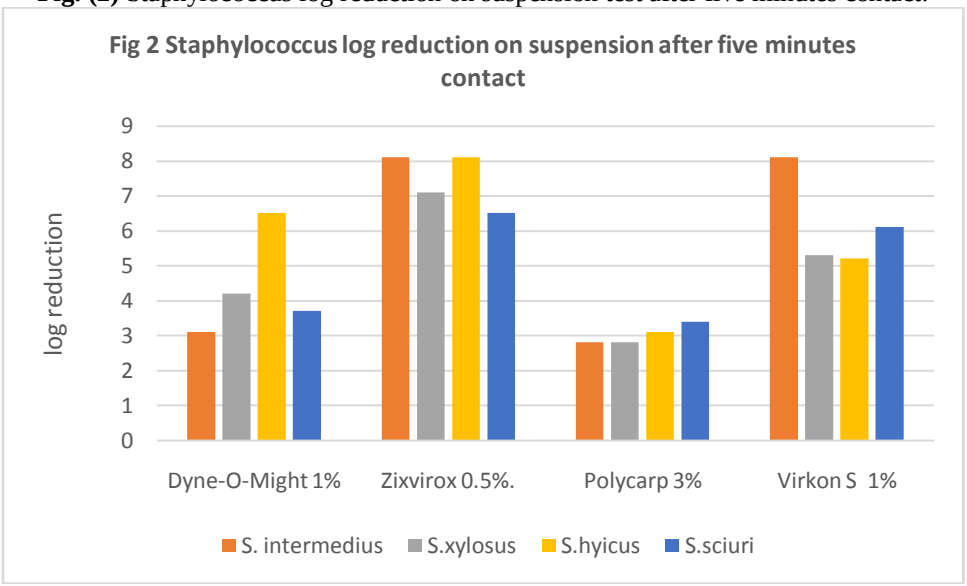
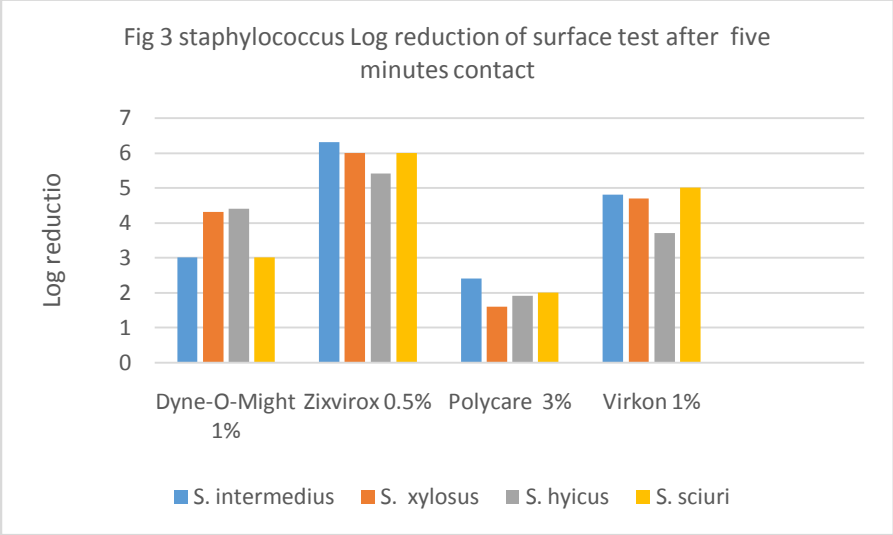



**Table (7)** Staphylococcus log reduction of Surface test after five minutes contact:

| | S. INTERMEDIUS | S. XYLOSUS | S. HYICUS | S. SCIURI |
|---|---|---|---|---|
| **DYNE-O-MIGHT 1%** | **3.0** | **4.3** | **4.4** | **3.0** |
| **ZIXVIROX 0.5%** | **6.3** | **6.0** | **5.4** | **6.0** |
| **POLYCARE 3%** | **2.4** | **1.6** | **1.9** | **2.0** |
| **VIRKON 1%** | **4.8** | **4.7** | **3.7** | **5.0** |

**Fig. (3)** Staphylococcus log reduction of Surface test after five minutes contact:




Staphylococcus aureus and coagulase-negative staphylococci (CNS) are common causes of bovine and caprine intermammary infections. S. aureus infections, which can be clinical or subclinical, frequently persist for a long time, and infected mammary glands thus serve as reservoirs from which the organism may spread to other cows within a herd and occasionally to other herds [21]. A main challenge in food industry is to avoid contamination of raw materials and products by pathogens and spoilage organisms by controlling of microorganisms on food contact surfaces such as milking machine, milking utensils and dairy equipment [22].

The results in tables (5&6) fig. (1&2) showed that the logarithmic reduction of average bacterial count of the four species of antibiotic resistance coagulase negative staphylococci tested up on addition of Dyne-O-Might 1% after contact time one-minute and after five minutes contact time was not achieved and not considered bactericidal according to the standard used except for Staphylococcus hyicus after five minutes contact time as the log reduction was 6.5. while other strains fail to response to this disinfectants this may be due to antibiotic resistance gene. Our results were coinciding to certain limit to those obtained by Boddie et al [23] and disagreed with McLure &Gordon [24] who found that, iodine exhibited a superior killing effect on 33 clinical isolates of methicillin-resistant Staphylococcus aureus when measured by rate of kill or final logarithmic reduction factors (LRFs ) achieved while Elisabeth et al. [25] mentioned that , iodine led to a log reduction of the viable cells of Staphylococcus epidermidis of >5 after incubation for 5 min . 0.2% povidone solution reduced methicillin-resistant Staphylococcus aureus under detection limits at 37, 24 hrs incubation and 7.5 % povidone-iodine, were most effective reducing MRSA under detection limits. (less than 10 CFU/ml) [26].

Concerning non chlorine releasing agent Zixvirox 0.5%, The required 5 log reduction in the suspension test was achieved even after short contact time one minute. These results agreed with Elisabeth et al. [25] who found that, Hydrogen peroxide, at a concentration of 3% and 5%, rapidly eradicate Staphylococcus epidermidis biofilms, whereas povidone-iodine is less effective, $H_2O_2$ demonstrates broad-spectrum efficacy against viruses, bacteria, yeasts, and bacterial spores. Leslie et al. [27] mentioned that, hydrogen peroxide-based teat disinfectant provided significant improvement in teat skin condition and no adverse effects on teat end condition. Peracetic acid is considered a more potent biocide than hydrogen peroxide, being sporicidal, bactericidal, virucidal, and fungicidal at low concentrations (<0.3%) [28]. These results may be due to the combination of peracetic acid and hydrogen peroxide was found to be synergistic. And that synergy was maintained with increasing contact time [29].

On the other hand, detergent agent like Polycare 3%was not considered bactericidal agent as the log reduction was lower than 5 even after five minutes contact time, Polycare is a surfactant used for cleaning purpose has low to moderate foaming ability and not considered bactericidal agent. Salah et al., [22] found that quaternary ammonium compound was not achieved the required log reduction even after 30 minutes' contact time when tested against staphylococcus aureus. So Polycare could be used as primary cleaning agent not effective disinfectant

Regarding addition of virkon S 1%, table (5) showed that, the log. reduction was greater than 5 after 5 minutes contact time for all tested strains except Staphylococcus intermedius the log reduction was 7.1 after one minute and 8.1 after 5 minutes.It could be referred to the resistant Antibiogramme they possess . Patterson et al. [30] concluded that, 4% peroxymonosulfate solution was successful in reducing counts of bacterial CFUs of S. aureus by > 99.9999%. Meanwhile Dunowska et al. [31] evaluate the efficacy of aerial disinfection using 1% virkon S and found that, The reduction of S. aureus counts ranged from 4.92 to 0.02 log (10).The

bactericidal activity of virkon may be due to the other ingredients which present with virkon like mallic acid ,these acid increase the acidity of virkon making it work better**.**

The results of surface test were cleared in table 7, fig. 3; the logarithmic reduction was somewhat lower than that obtained by suspension test. This may be due to the surface nature play the most important role and the destruction rate when the cells are in suspension is higher in this condition than when the microorganisms are settled on a surface [32], The variation of results between strain on applying surface and suspension test and within each test may have attributed to resistance antibiogramme which may decrease susceptibility or resistance to disinfectants.

## V. Conclusion

this work gives insight into the bactericidal activity of some commercially available disinfectants on some antibiotic resistant staphylococci strains, zixvirox gave excellent results even after one minutes contact times followed by virkon s while Dyne O Might and Polycare were not considered effective bactericidal agent and they may need more contact time to achieve the required log reduction, although all strains are staphylococcus antibiotics resistant strains they differ in their responses to different bactericidal agent as even within the same test the variation in results may be attributed to gene resistance.

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