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# A Study on Bacterial and Fungal Causes of Subclinical Mastitis in Dairy Cows

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## Abstract

A total of 120 random samples of milk were collected from dairy cows in some examined dairy farms located at different centers of Damietta governorate. California Mastitis Test (CMT), bacteriological and mycological examination were carried out for detection of positive cases of subclinical mastitis. The obtained results revealed that the most bacterial isolates were *Staph. aureus*, *Escherichia coli*, coagulase-negative staphylococci, *Streptococcus uberis*, *Corynebcterium bovis* and *Pseudomonas aeruginosa* with prevalence rate of 40%, 30%, 10%, 8.3%, 3.3% and 1.7%, respectively. While, the most frequently mycotic isolate was *Candidia albicans* with an incidence of 11.7%. Also, the obtained results revealed mixed infection of *Staph. aureus* and *C. albicans* in 4 samples and mixed infection of *E. coli* and *C. albicans* in 2 samples of cow's milk having subclinical mastitis. None of *Staph. aureus* strains which isolated from mastitic milk samples produce enterotoxins A, B, C, D or E. In Vitro, antimicrobial suceptiblity test of the bacterial isolates revealed that the most effective antibiotics were Ciprofloxacin, Levofloxacin, Cefataxime and Amoxycillin/Clavulanic acid. While, all bacterial isolates except *E. coli* were resistant to Cephalexin and Streptomycin. Also, all bacterial isolates except *Strept. uberis* were resistant to Erythromycin. The most effective antimycotic against *Candida albicans* was Fluconazole. The obtained results, conclusion, recommendation and preventive measures of subclinical mastitis were discussed.

**Key Words:** *Staph.* aureus, *Escherichia coli*, *Pseudomonas aeruginosa* , *Streptococcus uberis*, *Candidia albicans*, Mastitic Milk, Dairy Cow .

## Introduction

Mastitis is a global problem in farms of dairy cattle as it adversely affects animal health, quality of milk and economics of milk production and every country including developed ones suffering from financial losses. Mastitis usually occurs primarily in response to intramammary bacterial infection, but also to intramammary mycoplasmal, fungal, or algal infections. Clinical mastitis characterized by an increase in somatic cell count (SCC) in the milk, changes in milk composition and pathological changes in the

mammary tissues. While, udder tissues and milk appear apparently normal in subclinical mastitis. Diagnosis of subclinical mastitis is commonly based on cytological examination (SCC) and biochemical changes in milk but conformation must be applied by bacteriological mycological examination of milk (**Radostits et al., 2000**). Subclinical mastitis constitutes one of the important diseases of dairy animals, as infected quarters show no obvious symptoms and secrete milk that looks like apparently normal for long time, but contains high numbers of pathogenic microorganisms. Milk from cows with subclinical mastitis accidentally mixed into bulk milk enters food chains and posses a threat to human health. Therefore, milk from subclinical mastitis is considered a significant source of pathogens as well as its enterotoxins in raw milk may represents a serious health hazard and economic losses by decrease milk quality.

Thus, the present study was planned to investigate the prevalence of the most common bacterial and mycotic causes isolated from subclinically mastitic cows at Damietta governorate.

## Materials and Methods

### 1. Collection of samples:

A total of 120 random samples of suspected milk were collected from dairy cows in the examined dairy farms at Damietta governorate. All examined dairy cows were subjected to clinical examination by visual inspection, palpation of udder, beside the physical changes in the milk secreted from each quarter and California Mastitis Test (CMT) was carried out for all milk samples for detection of positive cases using Kerba test, Albert Kerbl GmbH Felizenzell 984428 Duchbach, Germany, according to method described by **Schalm et al. (1971)**. Each positive milk sample was collected from each quarter under complete aseptic conditions into two sterile screw capped McCartney bottles according to method described by Habashy et al. (2012). All collected milk samples were transported to the laboratory in an ice container for bacteriological and mycological examinations.

### 2. Bacteriological examination:

### 2.1. Isolation of causative bacteria:

All California Mastitis Test positive milk samples were thoroughly shacked and incubated at 37°C for 24 hours then centrifuged at 3000 r.p.m. for 20 minutes. The cream and supernatant fluid were discarded, while the milk sediments were cultured in nutrient broth and onto nutrient agar, MacConkey's agar, Lovine's eosin methylene blue (L-EMB) agar, Baird-Parker agar, Mannitol salt agar, Pseudomonas isolation agar and blood agar plates. All tubes and plates were incubated aerobically at 37°C for 24 hours. The developed colonies were picked up and subcultured for purification. The presence

of six or more bacterial colonies of the same type on the medium was considered to be significant and the sample was recorded as bacteriologically positive (**Batavani et al., 2003**).

**2.2. Identification of isolated bacteria:**

All pure colonies were identified morphologically by gram staining and biochemically by catalase test, oxidase test, motility test, haemolysis on blood agar, nitrate reduction , urea hydrolysis, screening on triple sugar iron agar, indole test, citrate test, VP test, MR test, coagulase test, growth at 45°C and various biochemical tests were done according to **Cruichshank et al. (1975); Carter and Cole (1990); Quinn et al. (1994) and Winn et al. (2006)**.

**2.3. Detection of staphylococcal entertoxins (SE):**

Staphylococcus aures strains which isolated from mastitic milk samples in the present study were examined for production of entertoxins A, B, C, D, and E by using Enzyme-Linked Immuno-Sorbant Assay (ELISA) according to (**Freed et al., 1982 and Lapeyre et al., 1988**).

**3. Mycological examination:**

**3.1. Isolation of yeasts:**

The sediment of each mastitic milk samples was cultured onto Sabouraud's dextrose broth, Sabouraud's dextrose agar contain 0.05 mg/ml chloramphenicol and malt extract agar acidified with lactic acid solution 10% at PH (3.4-4). The plates were incubated aerobically at 37°C for 5 days and examined daily for yeast isolation according to **Kwon-chung and Bennett (1992) and Fisher and Cook (1998).**

**3.2. Identification of isolated yeasts:**

The identification of isolated yeasts from mastitic milk samples was based on macroscopical, microscopical and physical properties according to techniques recommended by **Koneman et al. (1978), Barnett et al. (1983) and Kwon-chung and Bennett (1992).**

**Basis of clarifying subclinical mastitis:**

Mammary glands, without clinical abnormalities and with apparently normal milk, that both California Mastitis Test and bacteriologically or **mycologically positive were considered to have subclinical mastitis (Moawad and Osman, 2005)**.

**4. Antibiogram:**

Antibiogram of the isolated bacterial strains and the isolated strains of yeast from mastitic milk in this study was performed by using disc diffusion standard technique. The isolated strains of bacteria were tested against different types of antibiotic discs according to **Quinn et al. (1994) and Winn et al. (2006)**. While, the recovered strains of yeast were tested against antifungal discs according to **Lassa and Malinowski (2007)**.

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## Results and Discussion

The results recorded in Table (1) revealed the prevalence of the isolated strains of bacteria from the examined milk samples which collected from dairy cows suffering from subclinical mastitis during this study at Damietta governorate. The results showed that *Staph.* aureus was the main cause of subclinical mastitis in dairy cows and isolated from 48 mastitic milk samples representing 40% of total subclinical mastitic samples. All isolated strains of *Staph. aureus* were found coagulase-positive and none of these strains produced entertoxins A, B, C, D and E. Also, the obtained results revealed mixed infection of *Staph. aureus* and *C. albicans* in 4 samples representing 3.3% of total milk samples of dairy cows having subclinical mastitis. In addition, coagulase-negative Staphylococci were isolated from 12 samples representing 10% of total milk samples of dairy cows suffering from subclinical mastitis.

Higher prevalence rate of *Staph. aureus* in mastitic milk of cattle having subclinincal mastitis than our finding were recorded by **(Shitandi and Kihumbu, 2004; Sharma et al., 2010 and Sharma et al., 2012)**. While lower prevalence of *Staph. aureus* than our finding were recorded by **(Kalmus et al., 2011** and **Sztachanska et al., 2016)**. Higher prevalence rate of coagulase-negative staphylococci than our results were recorded by **(Shitandi and Kihumbu, 2004**; **Kalmus et al., 2011 and Sztachanska et al., 2016)**. While, lower prevalence rate of coagulase- negative staphylococci was reported by **Sharma et al., 2012**. Variations in the isolation rate may be due to number of samples, seasonal variation, treatment, herd health, hygienic conditions….etc.

*Staphylococcus aureus* is the most important and prevalent contagious mammary pathogen. It causes clinical and subclinical intramammary infection with serious economic loss and herd management problems in dairy cows (**Dego et al., 2002**). Prevalence of *Staph. aureus* infection may reach up to 82.22% in subclinical mastitis and up to 77.27% in clinical mastitis in dairy cows (**Sharma et al., 2012**). Most strains of *Staph. aureus* which isolated from mastitic milk are enterotoxigenic strains and toxic shock syndrome toxin-1 (TSST-1) producers. Therefore, it represent hazard to consumer's health, particularly to children, immune compromised patients and to elders (**Lim et al.,2004; Katsuda et al.,2005; Moon et al., 2007; Momtaz et al., 2010 and Guimaraes et al., 2011**).

The results recorded in Table (1) revealed that *Escherichia coli* was isolated from 36 mastitic milk samples representing 30% of total examined milk samples of cows suffering from subclinical mastitis. This high obtained incidence of *E. coli* isolation from mastitic milk of cows may be due to the poor hygienic measures where the muddy bedding materials cause a consequent udder infection from fecal contamination on such bedding glands. Lower incidence of *E. coli* isolation from milk samples of cows having

subclinical mastitis were recorded by **(Barbuddhe et al., 2001; Shitandi and Kihumbu, 2004; Sharma et al., 2010 and Sharma et al., 2012)**. Also, the obtained results in our study revealed mixed infection of *E. coli* and *C. albicans* in two samples representing 1.7% of total examined milk samples of cows having subclinical mastitis.

*Escherichia coli* is one of the most important environmental pathogens causing clinical and subclinical mastitis in dairy cattle and reported by many authors (**Sharma et al., 2010; Kalmus et al., 2011 and Nasef and Dawod, 2014**). *E. coli* mastitis is one of the major sources of economic losses in the dairy industry due to reduced milk production, treatment costs, discarded milk and occasional fatal disease (**Vangroenweghe et al., 2005**). In addition, Shiga toxin- producing *Escherichia coli* (STEC) isolates from mastitic milk were potentially pathogenic for human in that they belonged to serogroups associated with diarrhea and haemolytic-uraemic syndrome (**Lira et al, 2004 and Osman et al., 2012**).

The results recorded in Table (1) showed that *Streptococcus uberis* was isolated from 10 mastitic milk samples representing 8.3% of total examined milk samples of cows having subclinical mastitis. *Streptococcus uberis* is noncontagious (environmental) bacteria which may escape the natural defence mechanisms by multiplication along the streak canal (especially after milking), or by propulsion into the teat cistern by vaccum fluctuations at the teat end during milking. The infection occurs after bacteria gain entrance to mammary gland via the teat canal, (**Zhao and Lacasse, 2008**). *Streptococcus uberis* was isolated from mastitic milk samples of cattle during the course of clinical and subclinical mastitis by many authors (**Dego and Tareke, 2003; Shitandi and Kihumbu, 2004; Kivaria and Noordhuizen, 2007; Sharma et al, 2010; Vasie, 2009 and Kalmus et al., 2011**).

The results recorded in Table (1) showed that *Corynebacterium bovis* was isolated from 4 samples of mastitic milk representing 3.3% of total examined milk samples of cows having subclinical mastitis. *Corynebacterium bovis* primarily colonize the teat canal and are generally considered mildly pathogenic organisms. *Corynebacterium bovis* are capable of causing occasional udder infections with a mild increase in SCC and slight reduction in milk production. *Corynebacterium bovis* is isolated frequently from milk samples collected aseptically from dairy cows and generally considered a minor mammary gland pathogen or a commensal (**Pankey et al., 1985**).

*Pseudomonas aeruginosa* was isolated from only two samples of mastitic milk which representing 1.7% of total examined milk samples of cows having subclinical mastitis, Table (1). Higher prevalence rate of *Ps. aeruginosa* in subclinical mastitic milk was recorded by (**Seddek et al., 2000** and **Barbuddhe et al., 2001)**. *Pseudomonas aeruginosa* was implicated in many cases of both subclinical and clinical mastitis in

dairy cattle and its incidence in bovine mastitis may reach up to 60.4% (**Barbuddhe et al., 2001**). *Pseudomonas aeruginosa* was detected in the environment of dairy cows and isolated from raw milk. Also, this organism was isolated from milker's hands, moist areas of cow's body (udder and teats), drinking water, feeds, feces, milking equipments and walls and floors of the bran (**Otte et al., 1978**). In Egypt, several cases of food poisoning due to *Ps. aeruginosa* were traced to the consumption of raw milk and its products (**Ahmed et al., 1989**).

The results recorded in Table (2) illustrated the prevalence rate of isolated yeasts from the examined mastitic milk samples of cows having subclinical mastitis. The obtained results showed that *Candida albicans* was isolated from 14 mastitic milk samples which representing 11.7% of total examined milk samples of dairy cows having subclinical mastitis. Higher prevalence rate of *C. albicans* in mastitic milk was recorded by (**Kivaria and Noordhuizen, 2007 and Asfour et al., 2009**). In addion, the obtained results in our study revealed mixed infection of C. *albicans* and Staph. *aureus* in 4 samples of subclinical mastitic milk and revealed also mixed infection of *C. albicans* and *E. coli* in 2 samples of mastitic milk of dairy cows having subclinical mastitis. *Candida albicans* was isolated from mastitic milk samples of dairy cattle suffering from clinical and subclinical mastitis by many authers (**Kumar and Thakur, 2000; Senthilvelan et al., 2006; Kivaria and Noordhuizen, 2007 and Asfour et al., 2009**). Yeasts or their spores are very common in the dairy environment. Most animals show variable degrees of susceptibility to yeast infection. A common way for yeast to gain access into the udder endogenously in via the blood stream or exogenously by infusion of contaminated treatment preparations or using contaminated infusion equipment into the udder. Milking machine malfunction or poor milking technique are considered as possible routes that allow intramammary infection by yeasts. Yeasts do not respond to antibiotics and the abuse of antibiotics may worsen the signs of mastitis. Infection of the mammary glands by yeasts should be suspected when there was a history of unsuccessful antibiotic treatment which might aggrevate mycotic mastitis such as infection with Candida spp. which utilizes penicillin and tetracycline as a source of nitrogen (**Tarfarosh and Purohit, 2008**). Candida spp. are normal commensals of oral mucosa in human beings but increased exposure to affected animal and/or consumption of contaminated milk could be the cause for development of thrush in milkers. *Candida albicans* and its spores have the ability to survive pasteurization which is assumed to be of public health significance and has been indicated in cases of thrush in human beings (**Schmitt, 1971 and Tarfarosh and purohit, 2008**).

Results recorded in Table (3) showed susceptibility pattern (In Vitro) of the isolated bacteria from subclinial mastitic milk against different antibiotics. The obtained results revealed that the isolated strains of coagulase-positive *Staph. aureus* were sensitive to Ciprofloxacin 10 mcg at rate 40/48 (83.3%) and sensitive to both Cefotaxime 30 mcg and Levofloxacin 5 mcg at rate 36/48 (75%). Also, the isolated strains of *Staph. aureus* were susceptible to both Amoxycillin/Clavulanic acid 20/10 mcg and Doxycycline HCL 30 mcg at rate 24/48 (50%) and susceptible to both Neomycin 30 mcg and Gentamycin 10 mcg at rate 12/48 (25%). Also, the obtained results revealed that all strains of *Staph. aureus* which isolated from mixed infection of subclinical mastitis were sensitive to Ciprofloxacin 10 mcg, Cefotaxime 30 mcg, Levofloxacin 5 mcg and Amoxycillin/Clavulanic acid 20/10 mcg. While, the obtained results revealed that the isolated strains of *Staph. aureus* were resistant to each of Cephalexin 30 mcg, Erythromycin 15 mcg and Streptomycin 10 mcg. The obtained results revealed that all the isolated strains of coagulase-negative Staphylococci were very sensitive to both Ciprofloxacin 10 mcg and Levofloxacin 5 mcg at rate 12/12 (100%). Also, the obtained results showed that the strains of coagulase-negative Staphylococci were susceptible at rate 9/12 (75%) to each of Amoxycillin/Calvulanic acid 20/10 mcg and Cefotaxime 30 mcg, 6/12 (50%) to each of Doxycycline HCL 30 mcg and Neomycin 30 mcg and 3/12 (25%) to Gentamycin. The obtained results revealed the isolated strains of coagulase-negative Staphylococci were resistant to each of Cephalexin 30 mcg, Erythromycin 15 mcg and Streptomycin 10 mcg, Table (3).

The obtained results recorded in Table (3) showed that all the isolated strains of *Escherichia coli* were sensitive to Cefotaxime 30 mcg at rate 36/36 (100%). Also, the obtained results revealed the isolated stains of *Escherichia coli* were susceptible at rate 30/36 (83.3%) to each of Ciprofloxacin 10 mcg and Levofloxacin 5 mcg, 6/36(16.7%) to Amoxycillin/Clavulanic acid 20/10 mcg and susceptible at rate 18/36 (50%) to each of Cephalexin 30 mcg, Doxycycline HCL 30 mcg, Neomycin 30 mcg, Gentamycin 10 mcg and Streptomycin 10 mcg. Also, the obtained results revealed that all strains of *E. coli* which isolated from mixed infection of subclinical mastitis were sensitive to Cephalexin 30 mcg, Ciprofloxacin 10 mcg and Levofloxacin 5 mcg. While, the obtained results revealed all the isolated strains of *E. coli* were resistant to Erythromycin 15 mcg, Table (3).

The obtained results recorded in Table (3) revealed that the isolated strains of *Streptococcus uberis* were susceptible at rate 7/10 (70%) to each of Amoxycillin/Clavulanic acid 20/10 mcg, Ciprofloxacin 10 mcg, Doxycycline HCL 30 mcg, Erythromycin 15 mcg and Levofloxacin 5 mcg. Also, the isolated strains of Strept. *uberis* were susceptible at rate 5/10 (50%) to each of Cefotaxime 30 mcg and Neomycin

30 mcg, while all these strains were resistant to each of Cephalexin 30 mcg, Gentamycin 10 mcg and Streptomycin 10 mcg, Table (3).

The obtained results recorded in Table (3) showed all isolated strains of *Corynbacterium bovis* were sensitive at rate 4/4 (100%) to each of Cefotaxime 30 mcg, Ciprofloxacin 10 mcg and Levofloxacin 5 mcg; and were suceptible at rate 3/4 (75%) to each of Amoxycillin/Clavulanic acid 20/10 mcg and Doxycycline HCL 30 mcg. While, all the isolated strains of *Corynebacterium bovis* were resistant to each of Cephalexin 30 mcg, Erythromycin 15 mcg, Neomycin 30 mcg, Gentamycin 10 mcg and Streptomycin 10 mcg, Table (3).

The obtained results reported in Table (3) showed that the isolated strains of *Pseudomonas aeruginosa* were sensitive at rate 2/2 (100%) to Cefotaxime 30 mcg and were susceptible at rate 1/2 (50%) to each Ciprofloxacin 10 mcg, Doxycycline HCL 30 mcg and Levofloxacin 5 mcg. While, all the isolated strains of *Pseudomonas aeruginosa* were resistant to each of Amoxycillin/Clavulanic acid 20/10 mcg, Cephalexin 30 mcg, Erythromycin 15 mcg, Neomycin 30 mcg, Gentamycin 10 mcg and Streptomycin 10 mcg, Table (3).

The obtained results recorded in Table (4) revealed that all the isolated strains of *Candidia albicans* from subclinical mastitic milk of cows were sensitive at rate 14/14 (100%) to Fluconazole 10 mcg. Also, the isolated strains of *C. albicans* were susceptible to Nystatin 50 mcg at rate 10/14 (71.4%) and susceptible to Clotrimazole 10 mcg at rate 7/14 (50%), Table (4). Also, the obtained results revealed that all strains of *C. albicans* which isolated from mixed infection of subclinical mastitis were sensitive to Fluconazole 10 mcg and Nystatin 50 mcg.

**Conclusion and Recommendations:**

Data given by the obtained results from this work point out that the main cause of subclinical mastitis in dairy cows which isolated from its examined mastitic milk were contagious pathogen such as *Staph. aureus* and environmental pathogens such as *Escherichia coli*, Coagulase-negative staphylococci, *Strept. uberis*, *Corynebacterium bovis*, Pseudomonas *aeruginosa* and Candida *albicans*. Some of these organisms have public health significance and susceptible to antimicrobial drugs as well as it more resistant to other drugs. Therefore, to prevent and control of both subclinical and clinical mastitis in dairy cows, the following suggestion should be applied: routine examination of dairy animals and application of mastitic control programme for early detection of subclinical mastitis by California Mastitis Test (CMT), isolation of its causative organism by bacteriological and mycological examination and its treatment with the effective drug of choise. Prevention of appearance of new mastitic cases as well as

elimination of the cases of sever clinical symptoms. Strict hygienic measures should be applied for healthy udder, mastitic udder, teat dip, milking machine, dairy utensils and worker's hands. Educational programmes should be done for milk producers specially about the personal hygiene, environmental hygiene and proper feeding with balanced rations containing nutrient requirements from protein, fats, carbohydrates, vitamins, trace elements and anti-oxidants for dairy cattle.

**Table 1. Prevalence of isolated bacteria from mastitic milk of cows having subclinical mastitis.**

| **Isolated bacteria** | **Positive Samples** | |
|---|---|---|
| | **No.** | **%*** |
| ***Staph. aureus*** | **48** | **40** |
| ***Escherichia coli*** | **36** | **30** |
| **Coagulase-negative staphylococci** | **12** | **10** |
| ***Streptococcus uberis*** | **10** | **8.3** |
| ***Corynebacterium bovis*** | **4** | **3.3** |
| ***Pseudomonas aerginosa*** | **2** | **1.7** |

***: Calculated according to the total No. of examined samples (n. =120)**

**Table (2): Prevalence of isolated yeast from mastitic milk of cows having subclinical mastitis.**

| **Isolated yeast** | **positive samples** | |
|---|---|---|
| | **No.** | **%*** |
| ***Candidia albicans*** | **14** | **11.7** |

*: Calculated according to the total No. of examined samples (n. =120)

**Table (4): In Vitro susceptibility pattern of the isolated C. albicans against antimycotic drugs .**

| **Antimycotic drugs** | ***C. albicans* (n. =14)** |
|---|---|
| Clotrimazole 10 mcg | 7/14 (50%) |
| Fluconazole 10 mcg | 14/14 (100%) |
| Nystatin 50 mcg | 10/14 (71.4%) |

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**Table (3): In Vitro susceptibility pattern of the isolated bacteria against different antibiotics.**

| Isolated bacteria / Antibiotics | *Staph.aureus* "n.=48" | Coaguluse-negative staphylococci "n.=12" | *Escherichia coli* "n.=36" | *Strept.uberis* "n.=10" | *Corynebacterium bovis* "n.=4" | *Pseudomonas aeruginosa* "n.=2" |
|---|---|---|---|---|---|---|
| **Amoxycillin/ Clavulanic acid 20/10mcg** | 24/48 (50%) | 9/12 (75%) | 6/36(16.7%) | 7/10 (70%) | 3/4(75%) | 0 |
| **Cefotaxime 30mcg** | 36/48 (75%) | 9/12 (75%) | 36/36 (100%) | 5/10 (50%) | 4/4 (100%) | 2/2 (100%) |
| **Cephalexin 30mcg** | 0 | 0 | 18/36(50%) | 0 | 0 | 0 |
| **Ciprofloxacin 10mcg** | 40/48 (83.3%) | 12/12(100%) | 30/36 (83.3%) | 7/10(70%) | 4/4(100%) | 1/2 (50%) |
| **Doxycycline HCL 30mcg** | 24/48 (50%) | 6/12 (50%) | 18/36(50%) | 7/10 (70%) | 3/4 (75%) | 1/2 (50%) |
| **Erythromycin 15mcg** | 0 | 0 | 0 | 7/10 (70%) | 0 | 0 |
| **Levofloxacin 5mcg** | 36/48 (75%) | 12/12 (100%) | 30/36 (83.3%) | 7/10 (70%) | 4/4 (100%) | 1/2 (50%) |
| **Neomycin 30mcg** | 12/48 (25%) | 6/12 (50%) | 18/36(50%) | 5/10 (50%) | 0 | 0 |
| **Gentamycin 30mcg** | 12/48 (25%) | 3/12 (25%) | 18/36(50%) | 0 | 0 | 0 |
| **Streptomycin 10 mcg** | 0 | 0 | 18/36(50%) | 0 | 0 | 0 |

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