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# Prevalence of antibiotic resistant mastitis pathogens in dairy cows in Egypt and potential biological control agents produced from plant endophytic actinobacteria

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ABSTRACT

Dairy production is threatened by antibiotic resistant pathogens worldwide, and alternative solutions to treat mastitis are not available. The prevalence of antibiotic resistant strains is not well known in less developed countries. The prevalence of pathogenic bacteria and their resistance to 21 commercial antibiotics were studied in milk samples taken from 122 dairy cows suffering from the symptoms of mastitis in Egypt. The bacterial species were identified with molecular methods, and antibiotic resistance was studied with disc diffusion method. The prevalence of *Streptococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* were 30%, 17% and 3.5%, respectively. Most (90%) of the *S. aureus* strains showed resistance to penicillin whereas only 10% of the strains were resistant to oxacillin. Nearly half (40%) of *E. coli* strains showed resistance to streptomycin. Six *P. aeruginosa* strains showed resistance to several antibiotics, including ceftriaxone, enrofloxacin and levofloxacin. This points out that despite *P. aeruginosa* was not common, it should be followed up carefully. Potential biocontrol agents against antibiotic resistant mastitis bacteria were searched among 30 endophytic actinobacterial strains derived from wild medicinal plants. Three plants, namely *Mentha longifolia, Malva parviflora* and *Pulicaria undulata* were chosen for a more detailed study; their endophytic actinobacteria were used to prepare metabolic extracts. The crude metabolites of the actinobacteria were extracted with ethyl acetate. All metabolic extracts inhibited the growth of *S. aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *E. coli* and *P. aeruginosa in vitro*. The 16S rRNA sequence analysis revealed that the most efficient actinobacterial strains were two *Micromonospora* sp. and one *Actinobacteria bacterium*. We conclude that the combination of the metabolites of several endophytic actinobacteria derived from several medicinal plants would be the most efficient against pathogens. Different metabolite cocktails should be studied further in order to develop novel biocontrol agents to treat antibiotic resistant mastitis bacteria in dairy cows.


## 1. Introduction

Pathogenic bacteria are developing resistance to antibiotics all over the word. To cope with this problem, we need novel solutions, such as biocontrol agents. One group of potential organisms, which are known to produce antibacterial and antifungal metabolites, are actinomycetes. Endophytic actinomycetes as potential biocontrol agents are under intensive study worldwide, and generally considered as promising substitutes for chemical antibiotics (El-Shatoury

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et al., 2009; Elbendary et al., 2018; Gurung et al., 2009; Li et al., 2008; Matsumoto and Takahashi, 2017; Zhang et al., 2012).

One worldwide need for a novel biocontrol agent is a common infection in dairy cows, bovine mastitis. Mastitis have usually been treated with several commercial antibiotics; the frequent use of antibiotics have sometimes been inappropriate for a long time (Hogan and Smith, 2003; Rajala-Schultz et al., 2004; Robert et al., 2006; Vijayarathna et al., 2012). Therefore, mastitis therapy with antibiotics is suffering from the development of antibiotic resistant bacterial strains (Das et al., 2017; Klibi et al., 2018; Srednik et al., 2018; Wu et al., 2007). Despite a huge amount of studies all over the world, the prevalence of antibiotic resistant strains, and thus the urgency for novel biocontrol agents, is not well known in many less developed countries, such as in Egypt.

Mastitis has been treated with different ethno-veterinary medicines, i.e. for instance with traditional medicinal plants (Bullitta et al., 2018; Mushtaq et al., 2018). Recently, an *in vitro* study observed that the extracts derived from two medicinal plants, namely *Allium sativum* and *Bunium persicum*, were efficient against mastitis pathogens (Amber et al., 2018).

However, no great advancements in alternative treatments against clinical mastitis have been achieved; no recommendations about any alternative treatment was found by a recent review of Francoz et al. (2017). Therefore, novel solutions are still needed.

In this study, we first identified the most prevalent bacterial strains causing clinical bovine mastitis in Egypt and investigated their resistance to 21 commonly used antibiotics. Second, we produced metabolic extracts using plant endophytic actinobacteria and tested *in vitro* how efficiently they could inhibit the pathogenic bacteria causing mastitis. We aim to find biological and economic solutions to treat mastitis in dairy cows.

## 2. Materials and methods

### 2.1. Sample collection

The samples were collected randomly from a total of 122 lactating Holstein dairy cows showing palpable signs (hard and swollen quarter, kicking up on touching the udder and heat) from different dairy farms located in Ismailia Governorate, Egypt. Milk from each udder quarter was collected and examined visually for any anomalies (watery secretions, clots in milk, and blood-tinged secretions). Samples with any anomalies were collected aseptically; a total of 172 mastitis milk samples were transferred into laboratory and stored at 4 °C.

### 2.2. Isolation and identification of pathogens

Bacteria were isolated according to Islam et al. (2014) with some minor modifications. The loopful streaking technique was used to enrich the cultures, a loop of a milk sample was streaked in MacConkey agar and incubated aerobically at 37 °C for 24–48 h. The DNA was extracted from the isolates using DNA Purification Kit (Qiagen). The DNA was amplified with universal primers (5′CGATTCTGGAAATGGCAAAAG3′ and 5′CGTGATCAGCG GTGAC TATGAC3′) following the reaction mixture and thermal cycle described in Gashgari et al. (2019).

MRSA was identified according to Quinn et al. (2011). Briefly, when *S. aureus* strains showed antimicrobial resistance against oxacillin with the disc diffusion technique (below), the isolates were analyzed for *mecA* gene (genetic marker for MRSA indicated by specific bands at 310 bp in agarose gel. To identify pathogenic *E. coli* strains, three isolates of the most prevalent serotypes (O114, O128 and O158) of *E. coli* were detected for *phoA* gene (specific gene for *E. coli* strains) (Wenz et al., 2006).

### 2.3. Antibiotic resistance test

The antibiotic resistance of 87 pathogenic bacterial isolates were tested using the standard kibry Bauer method (disc diffusion technique). A total of 21 commonly used antibiotics (Oxoid) was tested (Table 1). The results are described as antibiograms where the bacterial strains are divided into resistant, intermediate and sensitive groups.

### 2.4. Actinobacterial extracts

A total of 30 endophytic actinobacterial extracts were tested for their efficiency to inhibit the mastitis pathogens. Actinobacteria had been isolated from wild medicinal plants of Sinai and reported in detail by El-Shatoury et al. (2006, 2013). The actinobacterial metabolites extracts, referred only metabolites hereinafter, were prepared as follows. First, the spore suspensions of the actinobacteria isolates were grown in 30 ml sub-cultured broth at 28 °C, 100 rpm for 7 days. The crude metabolites were extracted with ethyl acetate (1:1 v/v) for three successive times, with vigorous shaking for 30 min. The solvent layers were combined and concentrated under vacuum using rotary evaporator (HS2005S-N-Hahn Shin Scientific Co.) and re-dissolved in ethyl acetate to give a final concentration of 160 μg $mL^{-1}$.

The disc diffusion method with slight modifications (Mythili et al., 2018), was used to evaluate the antimicrobial activities of the metabolites. Isolated bacteria were inoculated on Muller Hinton agar plates (Himedia, India) ($10^7$ CFU $mL^{-1}$, 0.5 McFarland standard). The metabolites (10 μl) were impregnated in sterile discs (Whatman, 6 mm) that were placed on the plates. The plates were incubated at 37 °C for 18–24 h and the inhibition zones were recorded. Ethyl acetate saturated discs were used as negative controls. Standard antibiotic discs were used as positive controls; imipenem for *P. aeruginosa*, chloramphenicol for MRSA and *E. coli*, and oxacillin for *S. aureus*. All combinations of pathogens and metabolites were tested as duplicates.

Three metabolites that inhibited several pathogenic strains were measured for the minimum inhibitory concentration (MIC) using the broth dilution method according to Forbes et al. (2007). Pathogenic bacteria were cultured ($10^7$ CFU $mL^{-1}$) in the serial dilutions of the metabolites (range from 160 to 1.25 μg $mL^{-1}$) at 37 °C for 24 h. The minimum metabolite concentrations with no

**Table 1**
Frequency of bacterial species isolated from dairy cows suffering from clinical mastitis in Egypt.

| Bacterial species | No. of isolates | % | No. of infected animals | % |
|---|---|---|---|---|
| *S. aureus* | 51 | 29.7 | 46 | 37.7 |
| *E. coli* | 30 | 17.4 | 30 | 18.9 |
| *P. aeruginosa* | 6 | 3.5 | 6 | 4.9 |
| Minor growth[1] | 52 | 25.6 | 30 | 24.6 |
| No growth | 15 | 13.4 | 10 | 8.2 |
| Total | 172 | 100 | 122 | 100 |

[1] Not identified.

detectable pathogenic bacterial growth were considered as MIC. The absence of turbidity confirmed the absence of growth.

### 2.5. Identification of endophytic actinomycetes

Isolates that produced the most effective extracts against mastitis pathogens were identified. The 16S rRNA gene was amplified using the universal primers 27f & 1522r (Lane, 1991). The reaction mixture and the thermal cycles have been described in Gashgari et al. (2019). PCR products were purified and sequenced by Macrogen Inc. (Seoul, Korea, https://dna.macrogen.com/eng/index.jsp). The sequences were analyzed against NCBI database using the BLAST program. A phylogenetic tree was created using the Maximum Likelihood method based on the Kimura 2-parameter model (Tamura et al., 2004).

## 3. Results

### 3.1. Bacterial species identified in milk

The dominant bacterial species in milk were *S. aureus* and *E. coli*. They were isolated from 38% and 19% of the 122 cows, respectively (Table 2). *Pseudomonas aeruginosa* was isolated from 5% of the cows. Five isolates of *S. aureus* gave a positive result for *mecA* gene showing the specific bands at 310 bp, and were thus identified as MRSA (Fig. 2). From a total of 30 *E. coli* isolates, three isolates were detected for the phoA gene (a bands at 720 bp) (Fig. 3) indicating pathogenicity for these three isolates.

Out of the 51 *S. aureus* strains, 90% showed high resistance to penicillin (Fig. 1). Many *S. aureus* strains were resistant also to tetracycline (57% of the strains) and gentamicin (27%). In contrast, many *S. aureus* strains were sensitive to amoxicillin-clavulanic a (80%), erythromycin (84%), oxacillin (73%), and trimethoprim–sulphamethoxazole (84%). Out of the 30 *E. coli* strains, 40% showed resistance to streptomycin (Fig. 1). Resistance was observed also to ampicillin (33%), trimethoprim–sulphamethoxazole (23%), ceftriaxone (20%), cefepime (16%) and tetracycline (16%). High susceptibility of *E. coli* was observed to several antibiotics; amikacin, amoxicillin- clavulanic a, ciprofloxacin, and for neomycin. More than 60% of the strains were sensitive to these antibiotics.

Out of the six *P. aeruginosa* strains, five (83%) showed resistance to ceftriaxone, enrofloxacin and levofloxacin, and four (67%) strains showed resistance to streptomycin, tetracycline, aztreonam, cefepime, trimethoprim – sulphamethoxazole, ciprofloxacin and ceftazidime (Fig. 1). All isolates were susceptible to imipenem. However, high susceptibility (>50% of the strains) was observed only to four antibiotics, namely amikacin, chloramphenicol, gentamicin and piperacillin.

### 3.2. Actinomycetes

Each of the 30 actinomycete metabolites exhibited antimicrobial activity against at least one of the pathogens tested. Pathogen *S. aureus* was inhibited by all metabolites, except one derived from plant *Fagonia mollis* (Table 3). MRSA, *P. aeruginosa* and *E. coli* were each inhibited by most of the metabolites. Three of the metabolites showed antibacterial activity against almost all pathogens with wide inhibition zones (10–13.5 mm).

These most efficient metabolites had been derived from actinomycetes strains that had been isolated from plants *M. longifolia, M. parviflora* and *P. undulata.* The metabolites' MIC against different pathogenic bacteria varied from 80 to 160 $\mu g\ mL^{-1}$ (Table 3).

The phylogenetic analysis revealed that two most efficient actinobacterial strains showed close similarity to *Micromonospora* sp. (KP784800), *Micromonospora chokoriensis* strain B020 (KY858243). One species showed close similarity to *Actinobacteria bacterium* strain 650–37 (MG807525) (Fig. 4).

## 4. Discussion

All milk samples were originating from cows suffering from the symptoms of mastitis. However, only about 50% of the milk samples showed positive pathogenic bacteria cultures in our tests. This may be explained by the fact that *E. coli* induced clinical mastitis infections are often of very short duration. In such cases, milk samples may not show any positive pathogen cultures (Sears et al., 1993). The severe cases of mastitis showed that the Egyptian cows were infected most often by *S. aureus, E. coli* and *P. aeruginosa*. These pathogen species have been found to be responsible for clinical mastitis also elsewhere (Akram et al., 2013; Hegazi et al., 2014; Riekerink et al., 2008).

The knowledge about the prevalence of antibiotic resistant strains causing mastitis in dairy cows is fragmentary in less developed countries in Asia, Africa and South America. Published results show great variation in the antibiotic resistance. For instance, in Ecuador, all mastitis pathogens were susceptible several antibiotics recently (Amer et al., 2018). In Ethiopia instead, a relatively high prevalence of *S. aureus* was found; more than half of mastitis

**Table 2**
Inhibition of the metabolic extracts of actinobacteria derived from different plant species against four pathogenic bacteria. The species in bold were the most efficient and chosen for minimum inhibitory concentration analysis.

| Plant species | Zone of inhibition (mm; mean ± SD, $n = 3$) | | | |
|---|---|---|---|---|
| | *S. aureus* | MRSA | *E. coli* | *P. aeruginosa* |
| *Adiantum capillus venerris L* | 8 ± 0.0 | 6.5 ± 0.7 | 6.5 ± 0.7 | – |
| *Artimisia herba alba* | 9.5 ± 3.5 | 7.5 ± 1.4 | 7.5 ± 0.7 | 6.5 ± 1.4 |
| *Artimisia herba alba Asso* | 8 ± 0.0 | 6.5 ± 0.7 | 9 ± 0.0 | 6.5 ± 0.7 |
| *Asclepias sinacia* | 9 ± 1.4 | – | – | 6.5 ± 0.7 |
| *Ballota undulata* | 8.5 ± 0.7 | 9 ± 1.4 | 10.5 ± 3.5 | 10 ± 0.0 |
| *Echinops spinosissimus* | 8.5 ± 3.5 | 8.5 ± 3.5 | 8 ± 0.0 | – |
| *Fagonia mollis* | 7 ± 0.0 | 6.5 ± 0.7 | 6.5 ± 0.7 | 7 ± 1.4 |
| *Iphiona mucronat* | 10 ± 0.0 | 8 ± 0.0 | – | 9 ± 0.0 |
| *Lactuca orintalis* | 7 ± 1.4 | 9 ± 1.4 | 10.5 ± 0.7 | 8 ± 0.0 |
| ***Malva parviflora*** | 13 ± 0.0 | 13.5 ± 0.7 | 7.5 ± 0.7 | 7 ± 0.0 |
| ***Mentha longifolia*** | 8 ± 1.4 | 10 ± 0.0 | 10.5 ± 0.7 | 10 ± 1.4 |
| *Origanum syriacum* | 9.5 ± 0.7 | 6.5 ± 0.7 | 9 ± 1.4 | 10 ± 1.4 |
| ***Pulicaria undulata*** | 10 ± 0.0 | 10.5 ± 0.7 | 11 ± 1.4 | 9 ± 0.0 |
| *Solanum nigrium* | 7.5 ± 2.1 | 10 ± 0.0 | 9 ± 1.4 | 7.5 ± 0.7 |
| *Tecurium polium* | 9.5 ± 0.7 | 9 ± 2.8 | 10 ± 0.0 | 12 ± 0.7 |

MRSA = Methicillin-resistant *S. aureus*.

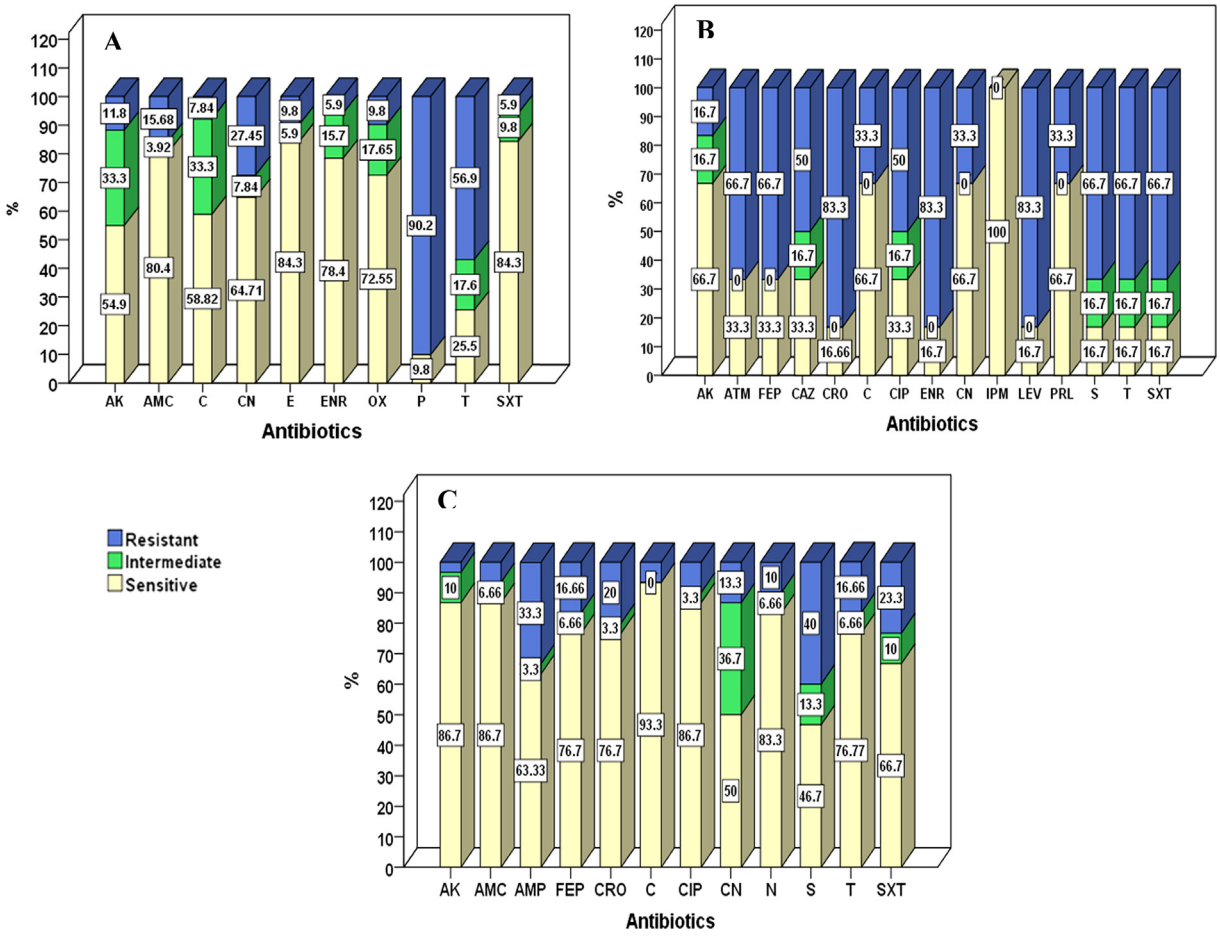


**Fig. 1.** Antibiogram of pathogenic bacteria S. aureus (A) P. aeruginosa (B) and E. coli (C) isolated from milk samples. Bacteria is divided into resistant, intermediate and sensitive groups according to the sensitivity to antibiotics.

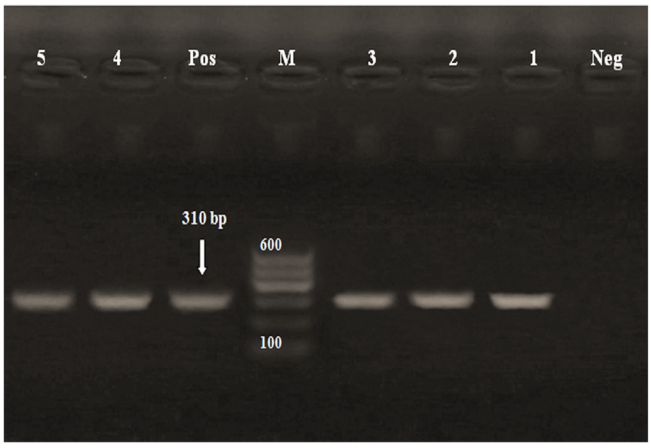


**Fig. 2.** Agarose gel electrophoresis of the amplified mecA gene PCR product (310 bp) for the five methicillin-resistant *S. aureus* (MRSA).

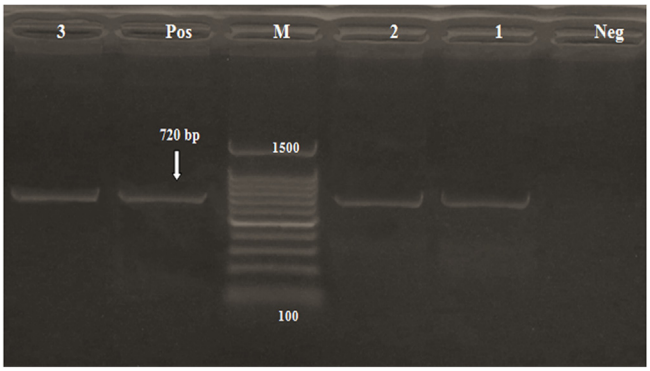


**Fig. 3.** Agarose gel electrophoresis of the amplified phoA gene PCR product (720 bp) for pathogenic *E. coli*.

infections were caused by *S. aureus* that was penicillin resistant, and resistant to several other antibiotics as well (Ayana et al., 2017). About two thirds of mastitis infections were caused by *S. aureus* and about 30% of them appeared to be MRSA in Saudi Arabia (Alzohairy, 2011). About half of the *Streptococcus* infections were resistant to all antibiotics tested in Syria (Al Laham and Al Fadel, 2013). One third of the cows suffering from mastitis had an antibiotic resistant strain in Egypt (Ahmed and Shimamoto, 2011). Each of the three pathogen species we observed, *S. aureus, E. coli* and *P. aeruginosa*, included antibiotic resistant strains. We observed a very high (90%) resistance of *S. aureus* against penicillin, however; only 10% of the strains were resistant to oxacillin.

We identified five MRSA strains in our milk samples. MRSA specifically has shown high resistance against penicillin and other β-lactam antibiotics as studied in many countries (Akindolire et al., 2018; Erskine et al., 2003, 2002; Guven et al., 2010; Sabour et al., 2004). MRSA strains have become a major problem because the strains are frequently resistant not only to most β-lactam antibiotics but also to many other antibiotics, including tetracyclines,

**Table 3**
Minimum inhibition concentration (MIC) of the actinomycetes extracts derived from plants against four pathogenic bacteria.

| Plant species | Bacterial species | MIC (μg mL$^{-1}$) |
|---|---|---|
| *Mentha longifolia* | *S. aureus* | 40 |
| | MRSA | 80 |
| | *E. coli* | 40 |
| | *P. aeruginosa* | 40 |
| *Malva parviflora* | *S. aureus* | 160 |
| | MRSA | 40 |
| | *E. coli* | 80 |
| | *P. aeruginosa* | 40 |
| *Pulicaria undulata* | *S. aureus* | 160 |
| | MRSA | 160 |
| | *E. coli* | 80 |
| | *P. aeruginosa* | 40 |

aminoglycosides, macrolides, and lncosamides. However, *S. aureus* still seemed to be sensitive to several antibiotics in Egypt. *S aureus* has high genetic diversity, which exacerbates the developing of its control agents (Akindolire et al., 2018).

Many studies have focused on *S. aureus*. However, some other, less frequent species could pose a great threat as well. We observed a rare occurrence of *P. aeruginosa*, six cases. However, all of them showed broad-spectrum resistance to several antibiotics. This indicates the importance to follow up the antibiotic resistance and the emergency in putting efforts to find alternative treatments for mastitis.

*Escherichia coli* strains have generally been shown to be relatively resistant against streptomycin (Srinivasan et al., 2007); 40% of our strains showed resistance to streptomycin. However, *E. coli* seemed to be sensitive to several commercial antibiotics. In contrast, *P. aeruginosa* and its resistance to several commercial antibiotics should be a matter of concern. Although *P. aeruginosa* was isolated only from some of our milk samples, it appeared to be resistant to several antibiotics commonly in use. The only effective antibiotic against *P. aeruginosa* infection was imipenem in our tests. *P. aeruginosa* has been found to be highly resistant to antibiotics in general (Nam et al., 2010; Ohnishi et al., 2011), and also in case of mastitis pathogens (Erskine et al., 2002; Tenhagen et al., 2006). However, this was the first study reporting its high antibiotic resistance in Egypt.

Novel strategies, such as vaccination, bacteriophage therapy, nanomedicines and genetical improvement of the cattle are all needed to solve the problem with antibiotic resistant mastitis pathogens (Basdew and Laing, 2011; Berni et al., 2013; Derakhshani et al., 2018; Martin et al., 2018; Pereira et al., 2011). However, especially in less developed countries, easier and economic local solutions, such as biocontrol agents are of great promise (Mushtaq et al., 2018; Nair et al., 2015). Biocontrol agents could be produced from traditional medicinal plants, which indeed have been studied to some extent (Baskaran et al., 2009; Doss et al., 2012; Mubarack et al., 2011; Tadele et al., 2010). The antimicrobial activity of several ethno-veterinary has been reported. Herbs, such as *Artemisia herb alba*, *Jasonia montana* and *Thymus vulgaris,* as well as the tree leaves of *Maesa lanceolata* and *Leucosidea sericea* have been shown to have antibacterial properties against mastitis pathogens (Adamu et al., 2014; Al Laham and Al Fadel, 2013; Zeedan et al., 2014). The extracts of actinobacteria isolated from farm soil appeared to be efficient against several pathogens, including *S. aureus* (Elbendary et al., 2018). However, few studies

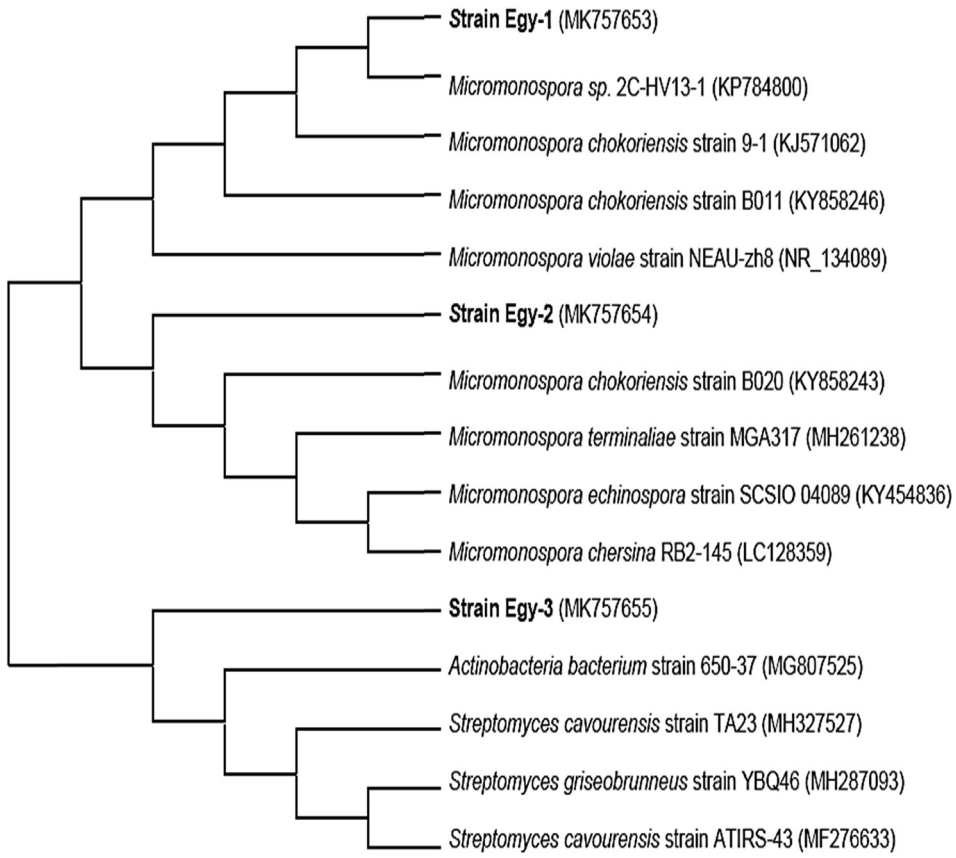


**Fig. 4.** Phylogenetic tree of ITS rDNA sequences of the endophytic actinobacteria used to prepare metabolic extracts against pathogenic bacteria causing mastitis in dairy cows. The tree was constructed by the neighbor-joining algorithm using the maximum composite likelihood model. Bootstrap percentages from 1000 replicates are shown.

report the antimicrobial activity of the endophytic bacteria isolated from ethno-veterinary plants (Nath and Joshi, 2015). Our approach was to use the medical plant species *Mentha longifolia, Malva parviflora* and *Pulicaria undulata* and isolate plant endophytic actinobacteria.

In our study, endophytic actinobacterial isolates showed various potential to inhibit pathogenic bacteria. Three of our actinobacterial strains indicated relatively high antibacterial activity *in vitro*. The minimum inhibitory concentration of the metabolic extract varied remarkably depending on the plant species and the pathogenic bacteria. Each pathogenic species seemed to be sensitive to at least one of the actinobacterial metabolite. Thus, a combination of different actinobacterial metabolites derived from different medicinal plants might be efficient against several pathogens, and should be studied further.

The actinomycetes species that had substantial antibacterial activity belonged to *Micromonospora* or *Streptomyces* genera. *Streptomyces* species are generally known to have antimicrobial properties against several pathogens, including MRSA (Han et al., 2018; Mohamed et al., 2018). *Micromonospora* genus has been shown to have antibacterial properties, especially *M. echinospora* is known to produce several broad-spectrum aminoglycoside derivatives (Choi et al., 2015; Kim et al., 2015; Tiwari and Gupta, 2012). Putting our findings together with previous reports on *Streptomyces* and *Micromonospora* genera, we suggest that these actinomycetes genera are of great promise in developing biocontrol agents against antibiotic resistant pathogenic bacteria.

## 5. Conclusion

We found several antibiotic resistant pathogenic bacteria causing bovine mastitis on dairy cows in Egypt. We also found endophytic actinobacterial species, which we isolated from medicinal plants, that might to be used to treat bovine mastitis. The dairy production in less-developed countries needs economic and local solutions to treat mastitis. Our observations are promising and the endophytic actinomycetes isolated from wild medicinal plants deserve further investigations to be developed as a practical biocontrol treatment against bovine mastitis on dairy cows. Future research could search for novel solutions by combining the metabolites extracted from several actinobacterial species isolated from several medicinal plants, and design a practical medical experiment.

## Declaration of Competing Interest

The authors declare that have no competing interests in this investigation.

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