Investigation of MLS_B and tetracycline resistance in coagulase-negative staphylococci isolated from the skin of Egyptian acne patients and controls

El-Mahdy, T.S. [*1,2]; Abdalla, S.[3]; El-Domany, R.[2] and Snelling, A.M.[1]
[1]Dept. of Biomedical Sciences, University of Bradford, UK [2]Faculty of Pharmacy, University of Helwan, Egypt
[3]Faculty of Pharmacy, University of Suez-Canal, Egypt
[*]Sata186@hotmail.com

Abstract: A total of 335 antibiotic-resistant coagulase-negative staphylococci (CNS) were isolated from face of 53 Egyptian acne patients, 13 dermatology staff and 36 controls. Prevalence of tetracycline resistant CNS was the most common with a rate of 87.3% of total population sampled. Acne patients treated with antibiotics were found to have significant higher risk of carrying erythromycin and clindamycin resistant CNS than patients not under treatment. Staff group was the most common cohort to carry multi-resistant CNS strains with a prevalence of 81.2%. Four erythromycin-resistance genes were screened for 43 CNS strains from patients. The most widely distributed determinants were *msr*(A) alone (48.8%), followed by *erm*(C) alone (39.6% strains) while both determinants together were accounted in 11.6% of the isolates. In addition, 48 non-duplicate tetracycline resistant CNS strains from patients were screened for the presence of four tetracycline resistance genes. Forty-seven of the isolates (97.9%) had *tet*(K) gene. *Tet*(L) gene was only found in four isolates (8.3%), from which three isolates were found to carry also *tet*(K) gene. This study revealed that the high carriage rate of *msr*(A) in our isolates suggests the effective therapy with clindamycin for most of erythromycin resistant CNS infections. In addition, the mechanism of tetracycline resistance in our isolates is mainly by active efflux and we might expect the success of treatment with minocycline in most of tetracycline resistant CNS from Egypt.
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1. Introduction:

Antibiotics remain the cornerstone of acne treatment. Whilst *Propionibacterium acnes* is being targeted, selective pressure is also exerted on other members of the commensal skin flora, including CNS. These bacteria may then act as reservoirs of resistance genes for more pathogenic strains or species. Several species of CNS are recognized as potential opportunistic pathogens, mainly causing nosocomial infections (Righter, 1987). CNS especially methicillin-resistant is one of the main causes of nosocomial blood stream infection in ICUs in Assiut University hospitals, Upper Egypt (Ahmed *et al.*, 2009)

Macrolide, lincosamide and streptogramin B (MLS_B) antibiotics are chemically distinct but share a similar mode of action. Three mechanisms have been involved in staphylococcal resistance to macrolides; target-site modification [encoded by *erm* genes] (Skinner *et al.*, 1983), active efflux [encoded by *msr*(A)] (Ross *et al.,* 1989), and rarely by drug inactivation (Weisblum, 1998). CNS strains carrying *erm* genes are cross-resistant to all MLS_B. However, strains carrying *msr*(A) gene are only resistant to MS_B antibiotics (Ross *et al*., 1990).

Bacterial resistance to tetracycline was mediated by: efflux proteins [encoded by *tet*(K) and *tet*(L) genes], ribosomal protection proteins [encoded by *tet*(M) and *tet*(O) genes], enzymatic inactivation of tetracyclines and target modification (Roberts, 2005). *Tet*(K) and *tet*(L) genes confer high level resistance to tetracycline but not minocycline. In contrast, *tet*(M) and *tet*(O) genes confer resistance to minocycline (Chopra & Roberts, 2001).

Erythromycin, clindamycin and tetracycline are widely used in Egypt to treat acne, and available over the counter, but it is not known how this is affecting the commensal flora. This study sought to determine the prevalence of resistant CNS, and genes responsible, isolated from Egyptian acne patients attending dermatology clinics and controls.

2. Materials and methods

Subjects

A total of 53 patients (23 male and 30 female) aged 15-29 years (average 20 years) attending two dermatology clinics in Cairo at:

Dermatology Clinic, Ain-Shams University Hospital and Cairo Dermatology Hospital, participated in this study. Thirteen dermatology staff (nurses and doctors) from the same dermatology clinics were also sampled. Patients were whether currently on or off treatment. Also 36 age-matched controls from the community were not suffering acne and no antibiotics taken in past six months. All patients, controls and dermatology staff were informed and gave their verbal and written informed consent to take part in this study. All participants in the study were asked to fill a questionnaire. The study was approved by the local Ethics Committees.

Sampling method

Cutaneous CNS isolates were collected from the face of Egyptian acne patients and controls according to the method applied by Ross *et al*. (2003). Applying firm pressure, the surface of the entire face was rubbed with a transport swab (Copan Italia, Brescia, Italy) moistened in sterile wash fluid (0.075 mol/L sodium phosphate buffer, pH 7.9) containing 0.1% Triton-X 100. Swabs were placed into tubes of Amies medium prior to transfer in the same day of sampling at 4^{o}C to Bradford University, UK and arrived after two days by experienced courier on two shipments.

Isolation and identification of resistant CNS

Swabs were used to inoculate Muller-Hinton (MH) agar plates containing breakpoint concentrations according to CLSI guidelines of 4 mg/L erythromycin, 2 mg/L clindamycin or 8 mg/L tetracycline as well as antibiotic-free control plates, which were always inoculated last. After 48 hours aerobic incubation at 37^{o}C, plates were inspected for growth. One representative isolate in colonies morphologically resembling staphylococci was chosen per plate but if more than one colony morphology was evident, a representative of each was selected for further study using Gram staining, coagulase test (Staphaurex, Remel, USA) and carbohydrate fermentation (method adopted from Kloos & Schleifer, 1975).

Selected coagulase-negative strains were also further identified using MASTRING *Staphylococcus* ID kit for identification of CNS (Mast, UK) as per manufacturer's protocol and PCR-ribotyping of staphylococci protocol. Eleven CNS reference strains were used, included *S. hominis* NCTC 11320; *S. warnari* NCTC 11044; *S. capitis* NCTC 11045; *S. epidermidis* NCTC 11047; *S. cohnii* NCTC 11041, *S. haemolyticus* NCTC 11042; *S. epidermidis* NCTC 2749; *S. aureus* NCTC 6571 (Oxford); *S. simulans* NCTC 11046; *S. xylosus* NCTC 11043 and *S. hyicus* sub. *chromogenes* NCTC 11530.

Antibiotics

Antibiotics were purchased from Sigma (Poole, U.K.) and were dissolved in water with the exception of erythromycin, which was dissolved in absolute ethanol.

Determination of MICs

MICs for the three antibiotics were determined by agar dilution on MH agar as described by CLSI using multipoint inoculator (Denley, Tech Ltd, Bolney Sussex, U.K). Type strain *S. aureus* NCTC 6571 (Oxford) was included as a susceptible control.

DNA preparation

A. For PCR detection of tetracycline and erythromycin resistance genes in CNS

Lysostaphin (50µl at 2 mg/mL cells suspended in 1X TE buffer) was used to weaken the cell walls and incubating at 37°C for up to 1 h. Genomic DNA was extracted twice with phenol/chloroform and precipitated by absolute ethanol as described by Eady *et al*. (1993).

B. For PCR-ribotyping of CNS

Using NET (10 mM Tris, 1mM EDTA, 10 mM NaCl)/Achromopeptidase (stock solution 10 units/µl, Sigma, code A3547) solution as described by Kobayashi *et al*.(1994).

PCR-ribotyping for species identification of CNS

The PCR reaction was performed as mentioned before by Jensen *et al*. (1993). A pair of primers was used within the 16S-23S rRNA spacer region. The PCR-ribotyping amplification patterns of CNS isolates were visually compared with those obtained for the reference strains.

The electrophoresis of PCR-ribotyping of CNS was carried out using 1X TBE buffer (89 mM Tris, 89 mM borate, 2mM EDTA, pH 8.3) and the gel was run at 100V for three hours in a large gel tank. All obtained fragments were visualized by ethidium bromide (Sigma) staining after gel electrophoresis using 2% agarose gels. The sizes of the PCR products were determined by comparing them with the migration of 100-bp DNA ladder (Fermentas).

PCR to investigate tetracycline and erythromycin resistance genes in CNS

Table 1 gives the primer sequences and PCR reaction conditions for each target gene. The PCR reaction was performed in a 20 µl volume; containing 1 µl DNA extract, 2 µl of 10x thermopol buffer

containing 2mM $MgSO_4$ (New England Biolabs, Ipswich, UK), 2µl of PCR nucleotide mix including 2 mM each of dNTP (New England Biolabs, UK), 0.25 µl of 0.1nm/µl each primer (Sigma Genosys, Ltd, London, UK) and 0.1 µl of Taq DNA polymerase (New England Biolabs, NEB), the volume for each PCR reaction was completed to 20 µl by molecular biology grade water (Eppendorf, Hamburg, Germany). All PCR reactions were started by an initial denaturation step at 94°C for 4 min, and ended by a final elongation step at 72°C for 5 min. All obtained fragments were visualized as mentioned before.

Table 1: Primer sequences and PCR conditions used to detect tetracycline and erythromycin resistance determinants in CNS

Resistance gene	PCR primer sequence 5'-3'	PCR reaction conditions	Amplicon Size (bp)	GenBank accession no.	Positive control/ Reference
erm(A)	**F**-GTTCAAGAACAAT CAATACAGAG **R**-GGATCAGGAAAA GGACATTTTAC	30 cycles (30s at 94°C; 30s at 52°C; 1 min. at 72°C)	421	K02987	*S. aureus* CW9/pSES29/ Leclercq *et al*., 1989
erm(B)	**F**-CCGTTTACGAAAT TGGAACAGGTAAAGGGC **R**-GAATCGAGACTT GAGTGTGC	As *erm*(A)	359	U35228	*S. intermedius* / Trieu-Cuot *et al*., 1990
erm(C)	**F**-GCTAATATTGTTT AAATCGTCAATTCC **R**-GGATCAGGAAAA GGACATTTTAC	As *erm*(A)	572	X54338	*S. aureus* RN4220/pE194/ Leclercq *et al*., 1989
msr(A)	**F**-GGCACAATAAGA GTGTTTAAAGG **R**-AAGTTATATCATG AATAGATTGTCCTGTT	30 cycles (1 min at 94°C; 1 min at 50°C; 90s at 72°C)	940	X52085	*S. aureus* RN4220/pUL5054/ Ross *et al*., 1990
tet(K)	**F**-GTAGCGACAATA GGTAATAGT **R**-GTAGTGACAATA AACCTCCTA	30 cycles (30s at 94°C; 30s at 55°C; 30s at 72°C)	360	S67449	*S. aureus* RN4220/ PVPF5/ Guay *et al*., 1993
tet(L)	**F**-TCGTTAGCGTGCT GTCATTC **R**-GTATCCCACCAAT GTAGCCG	35 cycles (1 min at 94°C; 1 min at 58°C; 90s at 72°C)	267	U17153	*Bacillus cereus* VPC 1214/ Burdett *et al*., 1982
tet(M)	**F**-AGTTTTAGCTCAT GTTGATG **R**-TCCGACTATTTAG ACGACGG	35 cycles (1 min at 95°C; 1 min at 50°C; 2 min at 72°C)	1862	M21136	*Enterococcus faecalis* fol / Nesin *et al*., 1990
tet(O)	**F**-AACTTAGGCATTC TGGCTCAC **R**-TCCCACTGTTCCA TATCGTCA	35 cycles (1 min at 94°C; 1 min at 50°C; 90s at 72°C)	515	Y07780	*Escherichia coli* DH5α/ Taylor *et al*., 1987

Assay for resistance phenotype pattern in erythromycin resistant CNS

Selected CNS strains demonstrating erythromycin resistance were screened for the MLS_B and MS_B phenotype as described by CLSI guidelines, 2007. Flattening of the zone around the clindamycin disc indicated an inducible MLS_B phenotype while constitutive MLS_B phenotype shows no inhibition zone around both discs. In contrast, the MS_B resistant isolates do not show flattening of the clindamycin zone next to the erythromycin disc.

3. Results and Discussion:

Demographics of study participants

More than half of the patients (62.2%) had acne from a period of 1-3 years. Twelve (22.6%) patients sampled had never used any specific acne treatment prior to the study. However, only 26.4% of patients were currently on any kind of acne therapy at the time of sampling. There was no significant difference between patient sex and severity of acne ($P > 0.05$).

Prevalence of skin colonization with antibiotic resistant CNS

Figure 1 shows the % of people in each cohort carrying antibiotic resistant CNS amongst their skin flora, as determined from the primary selective plates. The prevalence of tetracycline resistance was the most common amongst antibiotics tested with a rate of 87.3% of all cohorts sampled. The difference in prevalence of tetracycline resistance between patients, clinic staff, and controls was not significant ($P>0.05$). Patients on current or very recent antibiotic treatment were no more likely to carry tetracycline resistant strains (24 people of 45) than those using other or no medication (21 people of 45) ($P>0.05$).

In keeping with the observations of Miller *et al.* (1996) where they studied the staphylococcal resistance on the skin of acne contacts and controls, a majority of our controls carried staphylococcal strains resistant to tetracycline (95.1% of their controls *vs* 91.4% of controls in the current study), erythromycin (70.7% *vs* 66.7%) and clindamycin (24.4% *vs* 27.8%). In addition, prevalence of bacterial resistance to erythromycin was 95% for *S. epidermidis* strains isolated from acne patients in a French study (Dreno *et al*., 2001). These results are higher than those reported by Nishijima *et al*. (1994) in Japan and by Bouchami *et al*. (2007) in Tunisia with a percentage of 61% and 62%, respectively, but nearly similar to our current study (81.1%) in Egypt and to Forssman (1995) in Switzerland with a percentage of 100%.

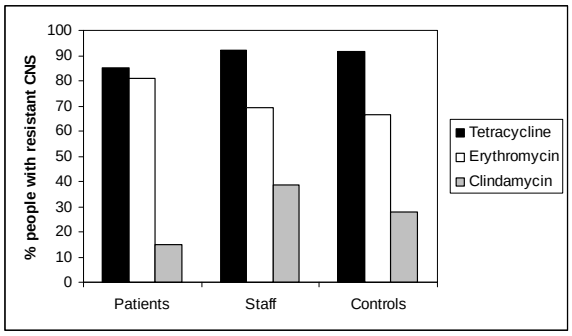

Figure 1: Prevalence of antibiotic resistant CNS amongst the cohorts tested

Egyptian acne patients treated with antibiotics were found to have a higher risk of carrying erythromycin and clindamycin resistant CNS ($P= 0.023$ and 0.036, respectively) than patients not under treatment. On the contrary, Dreno *et al*. (2001) showed that the use of previous or current treatment with erythromycin does not influence the frequency of resistant strains of *S. epidermidis*. This contradiction may be explained by the extensive use of both antibiotics in treatment of acne in Egypt.

Phenotypic and MIC profiles of antibiotic-resistant CNS

The susceptibilities of the 335 resistant isolates to the three antibiotics were determined by agar dilution. Table 2 illustrates the number of antibiotic-resistant CNS within the different cohorts.

Forty-seven strains of 117 (40.2%) carrying erythromycin resistance in patients had high-level resistance (MIC >1024 mg/L). Eight CNS strains of 116 (6.9%) from patients were found to have high-level resistance to tetracycline (MIC $\geq$ 256 mg/L). Staff group was the most common cohort to carry multi- resistant CNS strains with a prevalence of 81.2% of total strains from staff having resistance to two or three antibiotics. The incidence of multi-resistant CNS amongst isolates retained from patients and controls were 75.5% and 71%, respectively. All clindamycin resistant strains were also resistant to erythromycin.

Genetic diversity of erythromycin -resistant CNS

A total of 43 erythromycin resistant strains were retained from the 43 patients who were colonized with erythromycin resistant-CNS and identified to the species level. The PCR-ribotyping amplification patterns of CNS isolates were visually compared with those obtained for the reference strains.

Table 2: Number of antibiotic-resistant CNS strains and cross-resistance obtained from different cohorts

Antibiotic	No. of CNS strains resistant to antibiotic(s) / %			
	All cohorts (Total=335)	Patients (Total=163)	Controls (Total=124)	Staff (Total=48)
Tet	238 / 71	116 / 71.2	82 / 66.1	40 / 83.3
Ery	221 / 66	117 / 71.8	71 / 57.2	33 / 68.8
Clin	59 / 17.6	21 / 12.9	28/ 22.6	10 / 20.8
Cross-resistance to two Abs	151 / 45.1	70 / 42.9	60 / 48.4	21 / 43.8
Cross-resistance to three Abs	99 / 29.6	53 / 32.5	28 / 22.6	18 / 37.5

High level resistance to erythromycin was seen in 51.2% of the tested isolates (MIC >1024 mg/L) and all of these isolates harbor *erm*(C) gene (Table 3). The most widely-distributed erythromycin resistance determinants was *msr*(A). The expression of *erm*(C) was either inducible or constitutive. All CNS carrying only *msr*(A) were clindamycin susceptible and have low level resistance to erythromycin (MIC ≤ 128 mg/L). All strains carrying both *erm*(C) and *msr*(A) genes phenotypically express the inducible or constitutive MLS_B pattern. *S. simulans* and *S. epidermidis* were the major CNS species with *erm* mechanism (81.8%, 18 of 22 strains), in contrast, *S. haemolyticus* and *S. hominis* were the predominant *msr*(A) carrying species (61.5%; 16 of 26 strains) (Table 3).

Table 3: Genotypic and phenotypic distribution of erythromycin resistant CNS

Ery resistance gene(s)	No. of strains (%) (n=43)	Ery MIC range mg/L (mode)	Phenotype	CNS species
erm(A)	0	0	0	0
erm(B)	0	0	0	0
erm(C)	17 (39.6)	>1024 (>1024)	13 $iMLS_B$ 4 $cMLS_B$	[a]
msr(A)	21 (48.8)	16-128 (32)	21 non-inducible MS_B	*b*
erm(C)/ *msr*(A)	5 (11.6)	>1024 (>1024)	4 $iMLS_B$ 1 $cMLS_B$	*c*

$iMLS_B$: inducible MLS_B $cMLS_B$: constitutive MLS_B

[a]7 *S. simulans*, 6 *S. epidermidis*, 2 *S. haemolyticus*, 1 *S. hominis*, and 1 *S. saprophytics.*

[b]9 *S. haemolyticus*, 7 *S. hominis*, 2 *S. cohnii,* 1 *S. epidermidis,* 1 *S. saprophytics,* and 1 *S. simulans.*

[c]3 *S. epidermidis,* and 2 *S. simulans*

The available data corresponding to this study was compared in Table 4. Lina *et al*. (1999) found that macrolide resistance due to *msr*(A) was more prevalent in CNS (14.6%) than in *S. aureus* (2.1%). This *msr*(A) ratio in CNS is much lower than our finding that 60.5% of selected erythromycin resistant strains of CNS from Egypt have *msr*(A) gene alone or in combination with *erm*(C). Similarly to the current study, Bouchami *et al*. (2007) reported that the MIC of erythromycin varied between 32 and >1024 mg/L for isolates harboring *erm* genes and between 16 and 32 mg/L for those harboring *msr*(A).

The present study extends the data from previous studies that MLS_B resistance in CNS was caused most often by *erm*(C). Carriage of *msr*(A) is rarely seen in *S. aureus,* but seems to be more frequent in CNS (Lina *et al*., 1999). However, one study from USA (Fiebelkorn *et al*., 2003) reported that *msr*(A) gene was present in a high proportion of *S. aureus* isolates (36%), indicating that geographical differences may exist. It can be concluded from the present study that in our Egyptian CNS tested strains, clindamycin treatment should be considered as effective therapy due to the high carriage rate of the *msr*(A) gene by our isolates.

Table 4: Comparison of relevant studies on distribution of resistance genes *erm*(A), *erm*(B), *erm*(C) and *msr*(A) among isolates of CNS

Study	Location of isolates	Type of specimens	% of strains with					MLS_B Phenotype
			erm(A)	*erm*(B)	*erm*(C)	*msr*(A)	*erm* + *msr*(A)	
Eady *et al*., 1993	UK	Skin and clinical[a]	5.9	7.2	48	29.4	3.6	47% $iMLS_B$ 24% $cMLS_B$
Lina *et al*., 1999	France	Clinical	18	0.7	46.7	14.6	3.3	27.3% $iMLS_B$ 34.6% $cMLS_B$
Novotna *et al*., 2005	Czech Republic	Clinical	----	ND[b]	43[c]	53	16.3	16% $iMLS_B$ 20% $cMLS_B$
Martineau *et al*., 2000	Canada, China and France	Clinical	6.3	0.7	87.4	5.6	0	ND[b]
Thakker-varia *et al*., 1987	USA	Clinical	19	ND[b]	73.8	ND[b]	ND[b]	35.7% $iMLS_B$ 57.1% $cMLS_B$
Gatermann *et al*., 2007	Germany	Mostly clinical	5.3	2.3	65.6	23.6	2.4	25.6% $iMLS_B$ 51% $cMLS_B$
Aktas *et al*., 2007	Turkey	Clinical	8.9	6.4	78.2	11.5	3.8	20.6% $iMLS_B$ 57.8% $cMLS_B$
Bouchami *et al*., 2007	Tunisia	Clinical[d]	32	ND[b]	53	15	ND[b]	1% $iMLS_B$ 44% $cMLS_B$
Current study	**Egypt**	**Skin**	**0**	**0**	**39.6**	**48.8**	**11.6**	**39.5% $iMLS_B$ 11.6% $cMLS_B$**

$cMLS_B$: constitutive MLS_B $iMLS_B$: inducible MLS_B [a] from human and animal
[b] not determined [c] for *erm*(C) and *erm*(A) [d] from neutropenic patients

Genetic diversity of tetracycline-resistant CNS

A total of 48 non-duplicate tetracycline resistant CNS strains were chosen from patients and identified to the species level. Breakpoint of doxycycline and minocycline was 8 mg/L as set by CLSI. Forty-two strains (87.5%) had cross-resistance to doxycycline. The majority of isolates had doxycycline MICs in the range of 8-16 mg/L. None of the strains tested had resistance to minocycline.

All strains were screened for the presence of four tetracycline resistance genes; *tet*(K), *tet*(L), *tet*(M), and *tet*(O). Forty-seven of the isolates (97.9%) had *tet*(K) gene. *Tet*(L) gene was only found in four isolates (8.3%), from which three isolates were found to also carry *tet*(K) gene (Table 5). Similarly, Bismuth *et al.* (1990) from France reported that 97.6% of tetracycline resistant CNS carry *tet*(K), using DNA-DNA hybridization, which was detected in all of the species studied. Ardic *et al*. (2005) in Turkey reported that *tet*(K) genes were detected widely (42.9%) in CNS, whilst *tet*(M) genes were mainly seen in MRSA (50.0%). The frequency of *tet*(K) was much lower in Turkey than our study, but it is important to acknowledge that Ardic *et al.* (2005) had not selected the isolates on the basis of tetracycline resistance.

Table 5: Distribution of *tet* gene classes among CNS

tet resistance gene(s)	No. of strains (%) (n=48)	MIC range mg/L (mode)			CNS species
		Tetracycline	Doxycycline	Minocycline	
tet(K)	44 (91.7)	16->256 (64)	2-64 (8)	0.125-2 (0.25)	All[a]
tet(L)	1 (2.1)	64	16	0.25	*S. haemolyticus*
tet(K) / *tet*(L)	3 (6.2)	64-128 (128)	16	0.25-1 (0.5)	*S. haemolyticus* *S. epidermidis* *S. saprophyticus*
tet(M)	0	0	0	0	0
tet(O)	0	0	0	0	0

[a]13 *S. haemolyticus*, 11 *S. epidermidis*, 6 *S. hominis*, 4 *S. caprae*, 3 *S. cohnii*, 2 *S. saccharolyticus*, 2 *S. simulans*, 1 *S. saprophytics*, 1 *S. lantus*, and 1 *S. capitis.*

The finding that 47 of our 48 isolates (97.9%) had *tet*(K) gene and all isolates were minocycline susceptible comes in agreement with the documented phenomenon that the efflux proteins don't confer resistance to minocycline. This might be because minocycline is a lipophilic tetracycline derivative, which readily crosses the cytoplasmic membrane of the bacteria, possibly at quicker rate than the efflux pumps encoded by *tet*(K) or *tet*(L) can remove it (Speer *et al*., 1992).

In this study we illustrated that our CNS isolates from Egypt are resistant to tetracycline via the tetracycline efflux mechanism, and this is mainly due to the acquisition of the *tet*(K) gene and to a lower extent by *tet*(L). The *tet*(K) determinant appears to be widespread amongst CNS isolates in a broad range of countries, regardless of whether antibiotics can be purchased over the counter or not. Fortunately, the efflux mechanism of resistance to tetracycline does not confer resistance to minocycline. Consequently we might expect the efficient treatment with minocycline for the most tetracycline-resistant CNS from Egypt.

4. Conclusions and future work

The almost universal carriage of tetracycline resistant strains by controls may reflect the extensive use of the tetracyclines in dermatology and general medicine. Also our observations confirm that CNS isolates show an important reservoir of multi-resistance to the standard antimicrobials used for acne therapy likely due to prolonged use of antibiotics for acne therapy (Eady, 1998). The hospital dermatology staff can be an important source of transmission of resistant CNS from patient to patient. Strategies for reducing antibiotic use remain the major means of controlling resistance.

Cross-resistance between erythromycin and tetracycline was common amongst the skin isolates. Further investigations for the mobile genetic elements carrying erythromycin and tetracycline resistance genes were needed. These will elucidate if the high carriage rate of CNS isolates having both erythromycin- and tetracycline-resistance from Egypt is due to that these resistance genes were carried on the same transposons or plasmids or not.

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Corresponding author
El-Mahdy, T.S.
[1]Dept. of Biomedical Sciences, University of Bradford, UK [2]Faculty of Pharmacy, University of Helwan, Egypt
*Sata186@hotmail.com

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