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Advances in Microbiology, Infectious Diseases and Public Health

Gianfranco Donelli *Editor*

# Advances in Microbiology, Infectious Diseases and Public Health

Volume 2

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# Advances in Experimental Medicine and Biology

## Advances in Microbiology, Infectious Diseases and Public Health

Volume 901

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# Advances in Microbiology, Infectious Diseases and Public Health

Volume 2

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# Comparative evaluation of the antimicrobial activity of 19 essential oils

Naouel Chaftar, Marion Girardot, Jérôme Labanowski, Tawfik Ghrairi, Khaled Hani, Jacques Frère, and Christine Imbert

**Abstract**

In our research on natural compounds efficient against human pathogen or opportunist microorganisms contracted by food or water, the antimicrobial activity of 19 essential oils (EOs) was investigated against 11 bacterial species (6 Gram positive, 5 Gram negative) and 7 fungal species (2 dermatophytes, 1 mould, 4 yeasts) using microdilution assays. Five essential oils were obtained from Tunisian plants ($EO_{tun}$): *Artemisia herba-alba* Asso, *Juniperus phoenicea* L., *Rosmarinus officinalis* L., *Ruta graveolens* L. and *Thymus vulgaris* L., whereas others were commercial products ($EO_{com}$). Overall, *T. vulgaris* $EO_{tun}$ was the most efficient EO against both bacteria (Gram negative: MIC $\leq$ 0.34 mg/mL; Gram positive: MIC $\leq$ 0.70 mg/mL) and fungi (yeasts: MIC $\leq$ 0.55 mg/mL; mould: MIC = 0.30 mg/mL; dermatophytes: MIC $\leq$ 0.07 mg/mL). Two $EO_{com}$ displayed both acceptable antibacterial and antifungal potency, although weaker than *T. vulgaris* $EO_{tun}$ activity: *Origanum vulgare* $EO_{com}$ (bacteria: MIC $\leq$ 1.13 mg/mL, fungi: MIC $\leq$ 1.80 mg/mL), and *Cymbopogon martinii var. motia* $EO_{com}$ (bacteria: MIC $\leq$ 1.00 mg/mL, fungi: MIC $\leq$ 0.80 mg/mL). *Bacillus megaterium*, *Legionella pneumophila*, *Listeria monocytogenes and Trichophyton spp.* were the most sensitive species to

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both $EO_{com}$ and $EO_{tun}$. This study demonstrated the noteworthy antimicrobial activity of two commercial EOs and points out the remarkable efficiency of *T. vulgaris* $EO_{tun}$ on all tested bacterial and fungal species, certainly associated with its high content in carvacrol (85 %). These three oils could thus represent promising candidates for applications in water and food protections.

**Keywords**
Bacteria • Fungi • Essential oil • GC-MS • Antimicrobial activity • MIC

## 1 Introduction

Aerosols, water and food are among the main contamination pathways for human by pathogenic microorganisms. Even if it started a long time ago, research activity is still very active to find antimicrobial agents efficient to make these environments safe; in that way, there is a current research on natural products, and previous studies have investigated the interest of using EOs and their components in food preservation, such as vegetables, fruit juices (de Sousa et al. 2012; Espina et al. 2013) or meat (Fernandez-Pan et al. 2012; Hulankova et al. 2013). Essential oils could be used alone or combined with conventional biocidal molecules. The development of antimicrobial films may be also a technology to explore: the volatile molecules could be incorporated directly into the packaging material and would thus be released into the headspace surrounding the food (Appendini and Hotchkiss 2002; López et al. 2007; Muriel-Galet et al. 2012). EOs may be used also in water treatment to fight microbial pathogens (*Legionella* spp., *Pseudomonas* spp. . . .) occurring in spas, hammams, cooling towers. . .instead of the conventional chemical treatments which are currently not allowed by French legislation (Decree 89-369-06 June 1989) because they may change the initial composition of spa water. However, EOs are known for their hydrophobic properties which are an obstacle for water treatment. As a solution, EOs could be used as micellar solutions, as suggested by Edris and Malone (2012) who studied three EOs: clove bud (*Eugenia caryophyllata*), thyme (*Thymus serpyllum*) and oregano (*T. capitatus*). Their results contributed in developing formulations more thermodynamically stable for application in aqueous environment, cosmetics and pharmaceuticals (Edris and Malone 2012). Moreover, EOs could be used as a nanocapsular dispersion, as suggested by Shah et al. (2012). Consequently, EOs represent promising candidates for applications in water and food protection. Further work is needed to be able to implement all these strategies and to use EOs for the treatment of food or water. In this context, the aim of this study was to investigate the antibacterial and antifungal potency of EOs for The originality of our study is related to the high number of both EOs (19 EOs: 14 commercial EOs samples and 5 EOs obtained in our lab from Tunisian plants) and microbial species (18 species: 11 bacterial species – 6 Gram positive and 5 Gram negative – and 7 fungal species (2 dermatophytes, 1 mould, 4 yeasts) 93 investigated. The specific chemical composition of EOs was taken into account knowing that their composition may vary depending on the environment of the plant, the season and climate determining different chemotypes with changing therapeutic activities (Baatour et al. 2012; Bourgou et al. 2012; Hussain et al. 2008; Mighri et al. 2010a; Mohsen and Ali 2009; Rouis et al. 2012; Zouari et al. 2012). Studied EOs were selected because of their chemical interest

or their poor documentation. Tested bacterial species were mainly chosen because of their involvement in infections usually transmitted by food or water. Fungal species were especially chosen for their ability to contaminate human skin through moist surfaces.

## 2 Materials and Methods

### 2.1 Essential Oils

No endangered or protected plant species was included in this study. *A*erial parts of 5 plants (*Artemisia herba-alba* Asso*, Juniperus phoenicea* L.*, Rosmarinus officinalis* L.*, Ruta graveolens* L.*, T. vulgaris* L.) were collected twice from March to May 2010 and 2012 on private lands belonging to Tunisian co-authors who gave permission to collect the plants. *R. officinalis* was collected from the Tunisian northeast (region of Zaghouan), *A. herba-alba*, *R. graveolens* and *T. vulgaris* from the Sahel region (Sousse, Tunisia), and *J. phoenicea* from the Tunisian northwest (Aïn Drahem).

A voucher specimen of each plant was deposited at the Herbarium of the School of Pharmacy at the University of Poitiers (France). The plants were dried in the shade. EOs (indicated as $EO_{tun}$) were extracted by hydrodistillation for 4 h using a modified Clevenger-type apparatus and stored at 4 °C in tight vials in the dark until analysis as previously described (Chaftar et al. 2015).

14 commercial EOs (indicated as $EO_{com}$) were purchased from HYTECK laboratories (Clermont-Ferrand, France). These $EO_{com}$ were extracted from aerial parts of *Cymbopogon citratus* (India, Ref 0033)*; C. martinii var. motia* (India, Ref 0015)*; Mentha piperita Franco-Mitcham* (France, Ref 0039)*; Origanum vulgare* (Hongry, Ref 0182)*; R. officinalis* (Tunisia, Ref 0814)*; R. officinalis camphoriferum* (Spain, Ref 0019) and *T. vulgaris* (France, Ref 0226), leaves of *Cinnamomum tamala* (Nepal, Ref 0648)*; Eucalyptus globulus* (Portugal, Ref 0029)*; Melaleuca alternifolia* (Australia, Ref 0192) and *Syzygium aromaticum* (Madagascar, Ref 0073), needles of *Pinus laricio* (Corse, Ref 0531), zest of *Citrus sinensis* (Italy, Ref 0817) and seeds of *Trachyspermum ammi* (India, Ref 0021).

### 2.2 Terpenic Derivatives and Antimicrobial Agents

Carvacrol, thymol, amoxicillin and fluconazole were obtained from Sigma-Aldrich (Steinheim, Germany).

### 2.3 Gas Chromatography-Mass Spectrometry (GC/MS) Analysis

GC-MS analyses were performed as previously described (Chaftar et al. 2015). Briefly, these analyses were performed using a HP 6890 series chromatograph coupled to a HP 5973 mass selective detector. A Varian VF-5MS column (30 m, 0.25 mm, 0.25 μm) was used. The components were identified by comparing both their retention times with those of reference samples and determination of Kovats retention index, as well as by computer matching against commercial library mass spectra (NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library database version 2.0 d build Dec 2 2005) and mass spectra literature data. The quantification of each component was carried out by integrating the peak area of the chromatograms.

### 2.4 Organisms and Growth Conditions

In total, 18 strains potentially present in food or water were studied, including five strains of Gram negative species (*Klebsiella pneumoniae* 0502083 (laboratory collection), *Salmonella typhimurium* 124 (laboratory collection), *Escherichia coli* 27325 (laboratory collection),

*Pseudomonas aeruginosa* 910704 (laboratory collection) and *Legionella pneumophila* GC-A11 (laboratory collection)), six of Gram positive species (*Bacillus megaterium* F04 (laboratory collection), *Staphylococcus epidermidis* 567 (laboratory collection), *S. xylosus* 740 (laboratory collection), *S. haemolyticus* 694 (laboratory collection), *S. saprophyticus* 715 (laboratory collection) and *Listeria monocytogenes* EGDe) and seven of fungal species (four yeast species: *Candida albicans* ATCC 3153, *C. glabrata* IHEM 9556, *C. parapsilosis* ATCC 22019 and *Cryptococcus neoformans* (clinical strain); one mould specie: *Aspergillus fumigatus* ATCC 16424 and two dermatophyte species: *Trichophyton mentagrophytes* and *T. rubrum* (both clinical strains, hospital of Poitiers (France)).

Bacterial strains, except *L. pneumophila*, were grown in Brain Heart Infusion (BHI) (Bacto$^{TM}$) agar medium for 24 h at 37 °C. *L. pneumophila* was grown on Buffered Charcoal Yeast Extract (BCYE) agar medium supplemented with iron pyrophosphate (50 g/L) and L-cysteine (80 g/L) for 3 days at 37 °C in 5 % of $CO_2$. One colony was then suspended in buffered yeast extract (BYE) broth medium for *Legionella* or in BHI broth medium for other bacterial species. Optical density (OD) was adjusted to $10^6$ CFU/mL (OD measured at 600 nm; 1 OD $\approx 10^9$ CFU/mL), whatever species. Fungi were first grown on Sabouraud agar slants (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) for 48 to 96 h at 37 °C. Broth cultures were then prepared for each strain by transferring a loopful of this culture in RPMI /MOPS and the final concentration was adjusted to 0.5 MacFarland before dilution 1:1000 in RPMI /MOPS (*Candida* spp. and *C. neoformans*) or $10^5$ CFU/mL after filtration for *Aspergillus* and *Trichophyton* spp.

## 2.5 Minimal Inhibitory Concentrations

Minimal Inhibitory Concentrations (MIC) were performed by microdilution assay in 96 well-microplates. MIC was defined as the lowest EO concentration inhibiting visible microbial growth. Experiments were performed in triplicate and repeated twice in separate experiments. Preliminary tests were made using different solvents and culture media in order to select the best ones; results showed that acetonitrile and DMSO were the best choices for bacterial and fungal strains respectively. In our experimental conditions, these solvents did not influence the microbial growth compared to negative controls (data not shown). Regarding culture media, BHI was the most adapted for these experiments (data not shown).

Antibacterial activity was assessed as previously described (Verdon et al. 2008). The 19 EOs were first diluted in acetonitrile (50 %). The tested concentrations of EOs ranged between 0.17 mg/mL and 5.50 mg/mL except for *Legionella* (0.02–0.90 mg/mL). Microwells were inoculated with 100 µL of BHI or BYE medium supplemented with the tested strain at $10^6$ CFU/ml and 10 µL of each EO (serial concentrations), and incubated at 37 °C for 24 h except for *Legionella* (3 days in 5 % of $CO_2$). For each strain, growth controls without EO, acetonitrile controls and negative controls corresponding to the culture medium alone were prepared. Concerning bacterial strains amoxicillin was included as positive control (MIC ranging from $6{\cdot}10^{-6}$ mg/mL for *S. typhimurium* to >256 mg/mL for *K. pneumoniae* and *S. saprophyticus*. MICs were determined by the unaided eye and confirmed by a microplate analyzer reading (model Tecan-sunrise). The antifungal activity was evaluated according to the CLSI references M27-A3 and M38-A2 micromethods adapted protocols. EOs were first diluted 4:5 in DMSO. RPMI /MOPS was distributed in a 96-well microplate at 100 µL per well, except for the first column (200 µL/well); 4 µl of the tested oils were added in the first column and a serially dilution was performed. Then 100 µL of the preculture were added to each well. The tested concentrations of EOs ranged between 0.01 mg/mL and 8.80 mg/mL. The plate was incubated for 24–48 h (*Candida* spp.), 48–72 h (*C. neoformans* and *A. fumigatus*) or 96 h (*Trichophyton* spp.) at

27 °C, except for *Candida* spp. (37 °C). Fluconazole was used as positive controls. Growth controls without EO and DMSO controls were prepared.

## 3 Results and Discussion

Based on the existence of different chemotypes for each EO, potentially associated with various biological activities, the chemical composition of 19 EOs was investigated. The full composition of the five Tunisian EOs having been recently described (Chaftar et al. 2015), only the composition of commercial oils was detailed in Table 1 (components > 4 %).

The antimicrobial activity of those 19 EOs was also investigated against 11 bacterial species (6 Gram positive, 5 Gram negative) and 7 fungal species (2 dermatophytes, 1 mould, 4 yeasts) in order to bring out EOs with a large spectrum of activities suggesting a potential for food or water applications (Table 2).

Two of the most promising essential oils were *T. vulgaris* $EO_{tun}$ and *O. vulgare* $EO_{com}$. These oils had a close composition and were mainly composed of carvacrol (88.50 % and 66.89 %) and ρ-cymene (7.86 % and 21.20 %) respectively. They were highly active against all Gram positive (MIC ≤ 1.13 mg/mL), all Gram negative (MIC ≤ 0.34 mg/mL) bacteria and against all tested fungal species (MIC ≤ 1.80 mg/mL). *T. vulgaris* $EO_{tun}$ demonstrated activities close to those of fluconazole against *C. neoformans* and *T. rubrum* (MICs = 0.02 versus $1.60{\cdot}10^{-2}$ mg/mL).

*T. vulgaris* $EO_{com,}$ and *T. ammi* $EO_{com}$, both mainly composed of thymol (33.63 % and 49.29 %) and ρ-cymene (33.14 % and 40.75 %) respectively, were also active, but their efficiency only focused on two or four species of Gram positive (MIC ≤ 1.25 mg/mL) and two or one species of Gram negative (MIC ≤ 1.25 mg/mL) bacteria and on 6 species of fungi with MIC ≤ 1.80 mg/mL, dermatophytes being the most sensitive fungal strains (MIC ≤ 0.45 mg/mL). These high antifungal activities are consistent with results of Giordani and collaborators who have shown that EOs obtained from a thymol chemotype of *T. vulgaris* (French origin) were highly active against *C. albicans* (MIC 80 % = $1.60{\cdot}10^{-2}$ µL/mL) (Giordani et al. 2004).

Thymol and carvacrol being the major components of *O. vulgare* $EO_{com}$, *T. ammi* $EO_{com}$ and *T. vulgaris* $EO_{tun\ and\ com}$, their antimicrobial activity was evaluated against all bacterial strains except *B. megaterium* and *L. pneumophila*; results highlighted their interesting activity (carvacrol: MIC < 0.62 mg/mL, thymol: MIC < 0.31 mg/mL) except against *E. coli* (MIC = 2.50 mg/mL). MICs values were also important against the 3 *Candida* strains (MIC < 0.35 mg/mL for carvacrol and MIC = 0.10 mg/mL for thymol).

So, our results are in agreement with literature data showing the wide antimicrobial activity of thymol and carvacrol (Rattanachaikunsopon and Phumkhachorn 2010; Xu et al. 2008) and a limited occurrence in nature (Pinto et al. 2006); this therefore contributes to the great interest in essential oils containing these monoterpenes. They would act by disrupting and altering the morphogenesis of bacterial and fungal membranes (Braga et al. 2007; Trombetta et al. 2005). Due to their hydrophobic nature, these terpenes interact with the lipid bilayer of cytoplasmic membranes causing loss of integrity and leakage of cellular material such as ions, ATP and nucleic acid; cytoplasmic membranes are both permeabilized and depolarized (Trombetta et al. 2005; Xu et al. 2008). The high carvacrol content in *T. vulgaris* $EO_{tun}$ (88.50 %) and *O. vulgare* $EO_{com}$ (66.89 %) may explain the generally superior activity and broader spectrum of these oils compared to *T. vulgaris* $EO_{com}$ and *T. ammi* $EO_{com}$ which only contained 30 % and 49 % of thymol respectively. However, MICs of carvacrol seemed slightly higher than thymol ones, even if the differences observed between MIC values were not obvious for all tested species (Table 2). Furthermore, these four EOs also contained a significant proportion (in the range of 7.86–40.75 %) of the monoterpene ρ-cymene which is a precursor of carvacrol naturally present in these oils. It

**Table 1** Chemical composition of the 14 $EO_{com}$

| Component[a] | RT[b] (min) | KI[c] | *Cinnamomum tamala (%)* | *Citrus sinensis (%)* | *Cymbopogon citratus (%)* | *Cymbopogon Martinii var, Motia (%)* | *Eucalyptus globulus (%)* | *Melaleuca alternifolia (%)* |
|---|---|---|---|---|---|---|---|---|
| 1S $\alpha$ -pinene | 6.33 | 941 | 6.55 | – | – | – | – | 7.77 |
| Camphene | 6.78 | 958 | –[d] | – | – | – | – | – |
| $\rho$-Cymene | 8.83 | 1033 | 4.59 | – | – | – | 5.61 | 24.22 |
| D-limonene | 8.94 | 1037 | – | 83.62 | – | – | – | – |
| 1.8-cineol | 9.06 | 1041 | 13.28 | – | – | – | 88.91 | – |
| γ-terpinene | 9.77 | 1066 | – | – | – | – | – | 25.56 |
| *cis*-linalooloxide | 10.16 | 1079 | 11.72 | – | – | – | – | – |
| *trans*-linalooloxide | 10.62 | 1093 | 8.33 | – | – | – | – | – |
| Linalool | 10.97 | 1105 | 29.64 | – | – | – | – | – |
| Camphor | 12.42 | 1158 | 7.85 | – | – | – | – | – |
| D-*cis*-menthone | 12.65 | 1165 | – | – | – | – | – | – |
| D-*trans*-menthone | 12.91 | 1174 | – | – | – | – | – | – |
| Linalool epoxy | 13.02 | 1178 | 7.02 | – | – | – | – | – |
| Borneol | 13.13 | 1181 | – | – | – | – | – | – |
| Menthol | 13.29 | 1186 | – | – | – | – | – | – |
| $\alpha$-terpineol | 13.76 | 1201 | – | – | – | – | – | 12.04 |
| Neral | 14.95 | 1246 | – | – | 33.16 | – | – | – |
| Geraniol | 15.30 | 1258 | – | – | – | 77.40 | – | – |
| Geranial | 15.78 | 1275 | – | – | 49.15 | – | – | – |
| 3-menthene | 16.33 | 1293 | – | – | – | – | – | – |
| Thymol | 16.40 | 1296 | – | – | – | – | – | – |
| Carvacrol | 16.63 | 1304 | – | – | – | – | – | – |
| $\beta$-elemene | 17.68 | 1345 | – | 9.22 | – | – | – | – |
| Eugenol | 18.11 | 1361 | – | – | – | – | – | – |
| E-Patchenol | 18.27 | 1367 | – | 4.24 | – | – | – | – |
| Nerol acetate | 18.65 | 1380 | – | – | – | 13.07 | – | – |
| $\beta$-cadinene | 21.66 | 1497 | – | – | – | – | – | 17.76 |

[a]Compounds occupying more than 4 % are listed in order of their elution on the Varian VF-5MS column
[b]Retention time
[c]Kovats index
[d]Not detected

| *Mentha piperita Franco-Mitcham (%)* | *Origanum vulgare (%)* | *Pinus laricio (%)* | *Rosmarinus officinalis (%)* | *Rosmarinus officinalis camphoriferum (%)* | *Syzygium aromaticum (%)* | *Thymus vulgaris (%)* | *Trachyspermum ammi (%)* |
|---|---|---|---|---|---|---|---|
| – | – | 68.79 | 12.06 | 18.82 | – | – | – |
| – | – | – | 4.37 | 8.72 | – | – | – |
| – | 21.20 | – | – | – | – | 33.14 | 40.75 |
| – | – | – | – | – | – | – | – |
| 6.30 | – | – | 55.74 | 26.87 | – | – | – |
| – | – | – | – | – | – | 4.22 | – |
| – | – | – | – | – | – | – | – |
| – | – | – | – | – | – | – | – |
| – | – | – | – | – | – | – | – |
| – | – | – | 14.53 | 26.07 | – | – | – |
| 23.44 | – | – | – | – | – | – | – |
| 8.78 | – | – | – | – | – | – | – |
| – | – | – | – | – | – | – | – |
| – | – | – | 4.83 | – | – | – | – |
| 51.43 | – | – | – | – | – | – | – |
| – | – | – | – | – | – | – | – |
| – | – | – | – | – | – | – | – |
| – | – | – | – | – | – | – | – |
| – | – | – | – | – | – | – | – |
| 4.60 | – | – | – | – | – | – | – |
| – | 4.65 | – | – | – | – | 33.63 | 49.29 |
| – | 66.89 | – | – | – | – | 20.71 | – |
| – | – | – | – | – | – | – | – |
| – | – | – | – | – | 99.70 | – | – |
| – | – | – | – | – | – | – | – |
| – | – | – | – | – | – | – | – |
| – | – | – | – | – | – | – | – |

**Table 2** Minimal Inhibitory Concentrations (mg/mL) of 19 essential oils against the tested strains (five Gram negative

| | | Gram negative bacteria | | | | | Gram positive bacteria | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *K. pneumoniae* 0502083 (laboratory collection) | *S. typhimurium* 124 (laboratory collection) | *E. coli* 27325 (laboratory collection) | *P. aeruginosa* 910704 (laboratory collection) | *L. pneumophila* GC-A11 (laboratory collection) | *B. megaterium* F04 (laboratory collecti(on) | *S. epidermidis* 567 (laboratory collection) | *S. xylosus* 740 (laboratory collection) |
| *Artemisia herba alba* | Aerial parts | >4.50 | >4.50 | 2.25 | 2.25 | >0.90 | >4.50 | >4.50 | >4.50 |
| *Cinnamomum tamala* | Leaves | >4.50 | 2.25 | >4.50 | >4.50 | >0.90 | 2.25 | 4.50 | 4.50 |
| *Citrus sinensis* ($EO_{com}$) | Zest | >4.00 | >4.00 | >4.00 | >4.00 | >0.80 | >4.00 | >4.00 | >4.00 |
| *Cymbopogon citratus* | Aerial parts | >4.00 | >4.00 | >4.00 | >4.00 | 0.40 | 1.00 | 2.00 | 2.00 |
| *Cymbopogon martinii var. motia* | Aerial parts | 1.00 | 1.00 | 1.00 | 1.00 | 0.80 | 0.50 | 1.00 | 1.00 |
| *Eucalyptus globulus* | Leaves | >4.50 | >4.50 | >4.50 | >4.50 | >0.90 | >4.50 | >4.50 | >4.50 |
| *Juniperus phoenicea* | Aerial parts | >4.50 | >4.50 | 4.50 | 4.50 | <0.03 | 0.56 | >4.50 | 2.25 |
| *Melaleuca alternifolia* | Leaves | 2.25 | 2.25 | 2.25 | 2.25 | 0.90 | 2.25 | 4.50 | 4.50 |
| *Mentha piperita Franco-Mitcham* | Aerial parts | 4.50 | 2.25 | 2.25 | 2.25 | >0.90 | 2.25 | >4.50 | 2.25 |
| *Origanum vulgare* | Aerial parts | 0.30 | 0.30 | 0.30 | 0.30 | 0.12 | 1.13 | 1.13 | 1.13 |
| *Pinus laricio* | needles | 1.13 | 2.25 | 1.13 | 1.13 | 0.23 | 0.56 | 1.13 | 0.56 |
| *Rosmarinus officinalis* ($EO_{com}$) | Aerial parts | >4.50 | 4.50 | 4.50 | 4.50 | >0.90 | >4.50 | >4.50 | >4.50 |
| *Rosmarinus officinalis* ($EO_{tun}$) | Aerial parts | 5.50 | 5.50 | 2.75 | 2.75 | 0.55 | 2.75 | 5.50 | 5.50 |
| *Rosmarinus officinalis camphori-ferum* | Aerial parts | >4.50 | 4.50 | 4.50 | 4.50 | >0.90 | >4.50 | >4.50 | >4.50 |
| *Ruta graveolens* | Aerial parts | >4.00 | >4.00 | 4.00 | >4.00 | <0.02 | 0.50 | 4.00 | 2.00 |
| *Syzygium aromaticum* | leaves | 2.50 | 2.50 | 1.25 | 2.50 | 0.25 | 1.25 | >5.00 | >5.00 |
| *Thymus vulgaris* ($EO_{com}$) | Aerial parts | 2.50 | 2.50 | 1.25 | 2.50 | 0.12 | 0.31 | 5.00 | 1.25 |
| *Thymus vulgaris* ($EO_{tun}$) | Aerial parts | 0.34 | 0.34 | 0.34 | 0.34 | <0.03 | <0.17 | 0.70 | 0.34 |
| *Trachys-permum ammi* | Seeds | 2.25 | 2.25 | 2.25 | 2.25 | 0.12 | 0.56 | 4.50 | 1.13 |
| Carvacrol | | 0.31 | 0.31 | 0.31 | 0.62 | –[a] | – | 0.31 | 0.31 |
| Thymol | | 0.15 | 0.15 | 2.50 | 0.15 | – | – | 0.15 | 0.15 |
| Fluconazole | | – | – | – | – | – | – | – | – |
| Amoxicillin | | >0.25 | $6{\cdot}10^{-6}$ | $4{\cdot}10^{-3}$ | $3.20{\cdot}10^{-2}$ | $2{\cdot}10^{-3}$ | $6.40{\cdot}10^{-2}$ | $1.60{\cdot}10^{-2}$ | $16{\cdot}10^{-6}$ |

[a]Not determined

bacteria, six Gram positive bacteria, four yeasts, one mould and two dermatophytes)

| | | | Fungi | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *S. haemolyticus* 694 (laboratory collection) | *S. saprophyticus* 715 (laboratory collection) | *L. monoy-togenes* EGDe | *C. albicans* ATCC 3153 | *C. glabrata* IHEM 9556 | *C. parapsilosis* ATCC 22019 | *C. neoformans* (clinical strain) | *A. fumigatus* ATCC 16424 | *T. menta-grophytes* (clinical strain) | *T. rubrum* (clinical strain) |
| >4.50 | >4.50 | 2.25 | 7.20 | 7.20 | 7.20 | 3.60 | 7.20 | 7.20 | 1.80 |
| 4.50 | >4.50 | 1.13 | 3.60 | 3.60 | 7.20 | 1.80 | 7.20 | 0.45 | 0.45 |
| >4.00 | >4.00 | >4.00 | 6.40 | 3.20 | 3.20 | 1.60 | 1.60 | 0.10 | 0.05 |
| 2.00 | 2.00 | 0.50 | 0.80 | 0.80 | 1.60 | 0.40 | 0.80 | 0.05 | 0.01 |
| 1.00 | 1.00 | 0.50 | 0.40 | 0.80 | 0.80 | 0.40 | 0.80 | 0.20 | 0.20 |
| >4.50 | >4.50 | >4.50 | 7.20 | 7.20 | 7.20 | 7.20 | >7.20 | 0.45 | 0.90 |
| >4.50 | >4.50 | 0.30 | 3.60 | >3.60 | 3.60 | 3.60 | 3.60 | 3.60 | 3.60 |
| 4.50 | 4.50 | 2.25 | 3.60 | 3.60 | 3.60 | 1.80 | 1.80 | 0.45 | 0.45 |
| 4.50 | >4.50 | 1.13 | 1.8 0 | 3.60 | 3.60 | 1.80 | 0.90 | 0.11 | 0.03 |
| 0.56 | 0.56 | 0.30 | 0.90 | 0.90 | 1.80 | 0.90 | 0.90 | 0.11 | 0.06 |
| 1.13 | 1.13 | 0.56 | 3.60 | 0.45 | 1.80 | 0.23 | 1.80 | 0.23 | 0.23 |
| >4.50 | 4.50 | 2.25 | 7.20 | 7.20 | 7.20 | 7.20 | >7.20 | 3.60 | 1.80 |
| 5.50 | >5.50 | 0. 70 | 8.80 | 8.80 | 8.80 | 8.80 | >8.80 | 8.80 | 8.80 |
| 4.50 | 4.50 | 4.50 | 7.20 | 3.60 | 7.20 | 3.60 | >7.20 | 0.90 | 0.45 |
| >4.00 | 4.00 | >4.00 | 6.40 | >6.40 | >6.40 | 6.40 | 6.40 | 6.40 | 6.40 |
| 2.50 | 2.50 | 1.25 | 4.00 | 1.00 | 2.00 | 1.00 | 2.00 | 0.13 | 0.25 |
| 5.00 | 5.00 | 1.25 | 1.00 | 2.00 | 1.00 | 0.50 | 1.00 | 0.13 | 0.25 |
| 0.70 | 0.70 | 0.34 | 0.30 | 0.55 | 0.55 | 0.02 | 0.30 | 0.07 | 0.02 |
| 1.13 | 1.13 | 1.13 | 1.80 | 1.80 | 3.60 | 1.80 | 1.80 | 0.45 | 0.45 |
| 0.62 | 0.62 | 0.62 | 0.35 | 0.17 | 0.17 | – | – | – | – |
| 0.31 | 0.31 | 0.31 | 0.10 | 0.10 | 0.10 | – | – | – | – |
| – | – | – | $4{\cdot}10^{-3}$ | $3.10{\cdot}10^{-2}$ | $8{\cdot}10^{-3}$ | $1.60{\cdot}10^{-2}$ | - | $1.60{\cdot}10^{-2}$ | $1.60{\cdot}10^{-2}$ |
| $125{\cdot}10^{-6}$ | >0.25 | $16{\cdot}10^{-6}$ | – | – | – | – | – | – | – |

has been reported that ρ-cymene alone would not have bactericidal effect on bacteria but would enhance the antimicrobial activity of carvacrol (Burt 2004; Kisko and Roller 2005; Rattanachaikunsopon and Phumkhachorn 2010; Ultee et al. 2000).

$EO_{com}$ *C. martinii* var *motia* displayed quite high activities against all tested strains with MIC $\leq$ 1.00 mg/mL and was especially efficient against both dermatophytes (MIC = 0.20 mg/mL). $EO_{com}$ of *C. citratus* was active against the *L. pneumophila* strain (MIC = 0.40 mg/mL), two Gram positive bacteria: *B. megaterium* and *L. monocytogenes* (MICs = 1.00 and 0.50 mg/mL respectively) and all tested fungi (MIC $\leq$ 1.60 mg/mL), its highest activity being against dermatophytes (MIC $\leq$ 0.05 mg/mL) which is close to the fluconazole MIC value (MIC = $1.60{\cdot}10^{-2}$ mg/mL). Our results agreed with those of Bassolé et al. (2011) who studied some *C. citratus* EOs and also reported a high activity against *L. monocytogenes* and a poor one against *P. aeruginosa*. *P. aeruginosa* possesses an intrinsic resistance to a wide range of biocides which is associated with the nature of its outer membrane (Bassolé et al. 2011).

So, our results showed that *C. citratus* and *C. martinii* var *motia* EOs displayed different activities certainly related to their specific composition; geranial (citral A) (49.15 %) and neral (citral B) (33.16 %) were the leading components of $EO_{com}$ of *C. citratus* and geraniol (77.40 %) and nerol acetate (13.07 %) were the leading ones of *C. martinii* var *motia* oil. Citral is a monoterpene with known antifungal properties (Khan et al. 2012; Lima et al. 2012; Park et al. 2009). Citral was shown to be also active against *L. monocytogenes* (Friedman et al. 2002).

*P. laricio* oil was rather active against all tested species (MIC $\leq$ 1.80 mg/mL) except *S. typhimurium* and *C. albicans* (MIC $\geq$ 2.25 mg/mL). This oil mostly contained 1S α-pinene (68.79 %) and was highly active against *L. pneumophila*, *C. neoformans* and *Trichophyton* spp. (MIC = 0.23 mg/mL). Raman *et al.* reported, in agreement with our results, that α-pinene inhibited the growth of *S. epidermidis* (Raman et al. 1995).

1S α-pinene was also found in *M. alternifolia* EO, but at a lower concentration (7.77 %), in addition to γ-terpinene (25.56 %) and ρ-cymene (24.22 %). MICs of *M. alternifolia* $EO_{com}$ against all tested species were $\geq$2.25 mg/mL except against *L. pneumophila* (MIC = 0.90 mg/mL), *C. neoformans* and *A. fumigatus* (MIC = 1.80 mg/mL) and *Trichophyton* spp. (MIC = 0.45 mg/mL). Thus, compared to other tested oils, *M. alternifolia* $EO_{com}$ appeared poorly active. These MICs were in accordance with those described by Hammer et al. for a *M. alternifolia* EO also obtained from Australian plants (Hammer et al. 2012). Moreover, the obtained anti-*Candida* activity of *M. alternifolia* EO (MIC = 3.60 mg/mL) was higher than those described by Nenoff et al (MIC $\leq$ $4.40{\cdot}10^{-3}$ mg/mL) (Nenoff et al. 1996) and this oil has recently been shown to reduce *Candida* cell surface hydrophobicity (Sudjana et al. 2012). Here again, the composition was certainly determining; the γ-terpinene content was believed to more correlate with the inhibition of filamentation than to the antimicrobial activity (Vale-Silva et al. 2012), and as previously said, ρ-cymene would not have an antimicrobial activity. Dermatophytes were more susceptible (MIC = 0.45 mg/mL) than yeasts confirming the potential interest of *M. alternifolia* oil to treat tineas or ringworm (Pisseri et al. 2009).

Four bacterial species were susceptible to *S. aromaticum* $EO_{com}$ with MIC $\leq$ 1.25 mg/mL: *E. coli*, *L. pneumophila*, *B. megaterium* and *L. monocytogenes*. MICs obtained for dermatophytes were low (MIC $\leq$ 0.25 mg/mL) whereas other fungi displayed MICs of at least 1.00 mg/mL. The activity of *S. aromaticum* $EO_{com}$ could be related to eugenol (99.70 %). Antimicrobial and antioxidant properties of *S. aromaticum* EO have been already described by Chaieb et al. (2007) even if our results do not highlight remarkable antibacterial and antifungal activities. The antifungal activity of eugenol has been shown against *C. albicans* and *T. mentagrophytes*, the fungal cellular membrane

being probably its target (Chaieb et al. 2007). Moreover, Chami et al. (2005) have studied the fungicidal action of *S. aromaticum* EO on the yeast model *Saccharomyces cerevisiae* and showed that the surface of treated yeasts was significantly damaged (Chami et al. 2005). In addition, *S. aromaticum* EO would show some potential as a natural preservative or as a source of natural antioxidants for use in pharmaceutical applications (Chaieb et al. 2007).

*L. monocytogenes* was the most sensitive Gram positive bacterial species to *M. piperita Franco-Mitcham* $EO_{com}$ (MIC = 1.13 mg/mL), dermatophytes and mould being the most sensitive fungi (MIC $\leq$ 0.90 mg/mL), especially *T. rubrum* whose activity was close to that of fluconazole (MIC = 0.03 versus $1.60 \cdot 10^{-2}$ mg/mL). These results are generally in accordance with literature data. For example, Iscan et al. (2002) showed that an EO of *M. piperita* inhibited numerous human pathogenic microorganisms, *L. monocytogenes* being also the most sensitive species (MIC = 0.16 mg/mL). Regarding fungi, these authors only studied the activity of this *M. piperita* EO against *Candida* and the obtained MIC was 0.63 mg/mL, which is rather close to our results for *C. albicans* (1.80 mg/mL) (Iscan et al. 2002). GC-MS analyses showed that this EO contained menthol (51.43 %), D-*cis*-menthone (23.44 %) and D-*trans*-menthone (8.78 %).

In our study, the Italian *C. sinensis* $EO_{com}$ displayed a very poor antibacterial activity. On the whole, it was associated to MICs > 4.00 mg/mL and showed MICs ranging between 1.60 mg/mL and 6.40 mg/mL against fungi but was particularly efficient against dermatophytes (MIC $\leq$ 0.10 mg/mL). It should be noted that *C. sinensis* $EO_{com}$ was mainly composed of D-limonene (83.62 %).

*C. tamala* $EO_{com}$ was active against one bacterial strain: *L. monocytogenes* (MIC = 1.13 mg/mL) and 3 fungal strains: *C. neoformans* (MIC = 1.80 mg/mL) and *Trichophyton* spp. (MIC = 0.45 mg/mL). It was composed of linalool (29.64 %), *cis*-linalooloxide (11.72 %) and *trans*-linalooloxide (8.33 %) together with 1,8-cineole (13.28 %). Linalool could be mainly responsible for the activity of *C. tamala* EO as its antimicrobial effect was previously demonstrated (Pattnaik et al. 1997).

Both $EO_{com}$ and the $EO_{tun}$ of *R. officinalis* included in this study showed limited antimicrobial activity. *R. officinalis camphoriferum* $EO_{com}$ was only active against *Trichophyton* spp. (MIC $\leq$ 0.90 mg/mL). *R. officinalis* $EO_{com}$ was only and slightly active against *L. monocytogenes* (MIC = 2.25 mg/mL) and *T. rubrum* (MIC = 1.80 mg/mL). Finally, *L. pneumophila* (MIC = 0.55 mg/mL) and *L. monocytogenes* (MIC = 0.70 mg/mL) were the most susceptible bacterial species to *R. officinalis* $EO_{tun}$, other microorganisms showing MICs ranging between 2.75 and > 8.80 mg/mL. The major components of those three EOs were 1,8-cineole (>55 % for the two *R. officinalis* EOs and >25 % for *R. officinalis camphoriferum* $EO_{com}$), camphor (>14 %) and 1S α-pinene (>12 %) for both *R. officinalis* $EOs_{com}$ and borneol (>11 %) for *R. officinalis* $EO_{tun}$. However, it was previously shown that a *R. officinalis* EO (Chinese origin) was active against both Gram positive (*S. epidermidis, S. aureus, B. subtilis*) and Gram negative (*Proteus vulgaris, E. coli*) bacteria and *C. albicans* (Fu et al. 2007). The composition of this EO was slightly different from those we have studied: it was mainly composed of 1,8-cineole (27.23 %), α-pinene (19.43 %), camphor (14.26 %), camphene (11.52 %) and borneol (3.17 %) (Fu et al. 2007).

As *R. officinalis camphoriferum* $EO_{com}$, *E. globulus* $EO_{com}$ (Portugal origin) was only active against *Trichophyton* spp. (MIC $\leq$ 0.90 mg/mL). Our results do not agree with those of Damjanovic-Vratnica et al. (2011) who studied *E. globulus* EO (from Montenegro) and showed its high antimicrobial activity against some Gram positive and Gram negative bacteria (including *E. coli* and *K. pneumoniae*) and the yeast *C. albicans* with MICs ranging from 0.09 mg/mL to 1.57 mg/mL. However, in accordance with our results, this EO was poorly active against *P. aeruginosa* (MIC = 3.13 mg/mL)

(Damjanovic-Vratnica et al. 2011). This EO was rich in 1,8-cineole (88.91 %). *Eucalyptus* EO is recognized as GRAS (Generally Regarded as Safe) by Food and Drug Authority of USA and classified as non-toxic. The use of eucalyptus oil as a flavouring agent in foods (5.00 mg/kg), candies and confectionery items (15.00 mg/kg) has been approved. It is also used in soaps, detergents and perfumes preparations (Batish et al. 2008).

$EOs_{tun}$ from *R. graveolens* and *J. phoenicea* were generally poorly active against the tested species except *L. pneumophila* and *B. megaterium* (MIC $<$ 0.56 mg/mL), and especially against *L. monocytogenes* (MIC of *J. phoenicea* EO = 0.30 mg/mL). Up to now, *R. graveolens* EO was poorly studied even if *Ruta* species are a source of active natural products (some coumarins and alkaloids) (Meepagala et al. 2005; Stashenko et al. 2000). Some data related to the antibacterial properties of *J. phoenicea* EO (from Morroco) are available and suggest its weak activity against gram negative bacteria (including *E. coli* and *P. aeruginosa*; MIC $>$ 30.00 µL/mL) and moderate activity against Gram positive bacteria (including *L. monocytogenes*; MIC = 1.00 µL/mL) (Ait-Ouazzou et al. 2012). *J. phoenicea* $EO_{tun}$ was mainly composed of isoborneol (20.91 %) and 1S α-pinene (18.30 %).

*A. herba-alba* $EO_{tun}$ was the less active EO with activity ranging between $>$ 0.90 and 7.20 mg/mL. This EO was mainly composed of α-thujone (36.38 %), β-thujone (22.24 %) and camphor (19.12 %) (Chaftar et al. 2015). Our results do not agree with those of Mighri et al. (2010b) who studied four species of Tunisian *A. herba-alba* and described their relevant antimicrobial activities. However, any comparison is difficult because only four bacterial studied species were in common (*E. coli*, *S. typhimurium*, *C. albicans* and *C. glabrata*) (Mighri et al. 2010b).

Among all the studied EOs, the one with the highest activity was *T. vulgaris* $EO_{tun}$ which displayed low MIC values against Gram negative (MIC $\leq$ 0.34 mg/mL) and Gram positive (MIC $\leq$ 0.70 mg/mL) bacteria and fungi (dermatophytes, mould and yeasts; MIC $\leq$ 0.55 mg/mL). The most active $EOs_{com}$ against both bacteria and fungi were those obtained from *O. vulgare* and *C. martinii* var *motia* and, at a lower level, *C. citratus*, *P. laricio*, *T. vulgaris* ($EO_{com}$) and *T. ammi*. Their composition was specific and it was impossible to identify one or more shared components that could surely explain the presence of such an antibacterial and antifungal activity.

Regarding bacteria, *B. megaterium* and *L. monocytogenes* were the most susceptible of the tested Gram positive ones. The last one had MIC $\leq$ 0.56 mg/mL for four $EOs_{com}$ (*O. vulgare*; *P. laricio*; *C. citratus*; *C. martinii* var *motia*) and 2 $EOs_{tun}$ (*J. phoenicea* and *T. vulgaris*). *B. megaterium*, which is susceptible to amoxicillin (MIC = $6.40{\cdot}10^{-2}$ mg/mL), was also inhibited (MIC values $\leq$ 0.56 mg/mL) by four $EOs_{com}$ (*C. martinii* var *motia*, *P. laricio*, *T. vulgaris* and *T. ammi*) and four $EOs_{tun}$ (*C. sinensis*, *J. phoenicea*, *R. graveolens* and *T. vulgaris*) (Table 2). *L. pneumophila* strain, previously shown to be inhibited by amoxicillin (MIC $\leq 2{\cdot}10^{-3}$ mg/mL), was the most susceptible Gram negative strain (MIC $\leq$ 0.40 mg/mL for nine of the 19 tested EOs), *R. graveolens*, *T. vulgaris* and *J. phoenicea* $EOs_{tun}$ being the most active oils (MIC $\leq$ 0.03 mg/mL) against this species (Table 2).

Concerning fungi, the studied dermatophytes (*T. mentagrophytes* and *T. rubrum*), which were strongly inhibited by fluconazole (MIC = $1.60{\cdot}10^{-2}$ mg/mL), corresponded to the most sensitive strains and had MICs $\leq$ 0.25 mg/mL for eight $EOs_{com}$ (*C. sinensis*, *C. citratus*, *C. martinii* var *motia*, *S. aromaticum*, *M. piperita Franco-Mitcham*, *O. vulgare*, *P. laricio* and *T. vulgaris*) and MIC $\leq$ 0.07 mg/mL for *T. vulgaris* $EO_{tun}$.

## 4 Conclusion

The antimicrobial activity of 19 EOs was investigated and their chemical composition was taken into account. Results confirmed the potential interest of EOs as antimicrobial agents.

Among the tested EOs, *T. vulgaris* $EO_{tun}$ showed the widest spectrum of activity and, generally, the lowest MICs. This EO was mainly composed of carvacrol which was shown to have a stong and wide spectrum of antimicrobial activity. *O. vulgare* and *C. martini var. Motia* $EOs_{com}$ also displayed a wide spectrum of activity; they were mainly composed of carvacrol and geraniol respectively. Due to their activity against microbial species, these three EOs could thus represent promising candidates for applications in water and food protections. In addition, the significant anti-*Legionella* and anti-*Listeria* activity of most of the tested EOs constitutes a promising way to fight against the *Legionella* and *Listeria* risk, particularly in the case of recreational water and food respectively. Due to the fact that dermatophytes were the most susceptible fungi to both commercial and Tunisian EOs, these EOs may have also an interest in cosmetic industries, as components of some ointments or creams.

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**Conflict of Interest** The authors declare that they have no competing interests.

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# Advances in Microbiology, Infectious Diseases and Public Health: Refractory *Trichophyton rubrum* Infections in Turin, Italy: A Problem Still Present

Vivian Tullio, Ornella Cervetti, Janira Roana, Michele Panzone, Daniela Scalas, Chiara Merlino, Valeria Allizond, Giuliana Banche, Narcisa Mandras, and Anna Maria Cuffini

**Abstract**

Dermatophytosis caused by *Trichophyton rubrum* is the most common cutaneous fungal infection in industrialized countries and worldwide with high recurrence and lack of treatment response. In addition, patients with cutaneous and concurrent toenail lesions are often misdiagnosed and therefore treated with an inappropriate therapy. In this study, we evaluated five previously misdiagnosed cases of *T.rubrum* chronic dermatophytosis sustained by two variants at sites distant from the primary lesion. Our patients were successfully treated by systemic and topical therapy, and 1 year after the end of therapy follow-up did not show any recurrence of infection.

Our data indicate that the localization of all lesions, the isolation and the identification of the causative fungus are essential to establish the diagnosis and the setting of a correct therapeutic treatment to avoid recurrences.

**Keywords**

*Trichophyton rubrum* • Chronic dermatophytosis • Misdiagnosis

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Chronic dermatophytosis is a condition in which the clinical symptoms persist for more than 1 year with episodes of exacerbation and remission (Hay 1982; Zaias and Rebell 2003; Prasad et al. 2005). The main etiologic agent is *Trichophyton rubrum* responsible for 90 % of chronic infections (Di Chiacchio et al. 2014; Nenoff et al. 2014). Chronicity is probably related both to fungal cell wall components, such as mannan, that play an important role in the process of down-modulation of cell-mediated immune response of the host and to a lack of treatment response (Blake et al. 1991; Sato and Tagami 2003; Waldman et al. 2010). Patients with cutaneous and concurrent toenail lesions are often misdiagnosed and, therefore, treated with an inappropriate therapy (Larruskain et al. 2005).

In this study, we evaluated previously misdiagnosed cases of *T.rubrum* chronic dermatophytosis in five patients admitted to the Medical Sciences Department, University of Torino (Italy), through an investigation of clinical and mycological infection aspects.

**Case 1** A 42-year old male, born in Ecuador, reported a 7-year history of itchy and squamous lesions on the soles, toenails, palms and the nail plates, before arriving in Italy (Fig. 1a–d). Despite therapies with topical antibacterial agents in his native country, the patient had extensive erythema with painful papules, pustules and crusts in the chin and beard (Fig. 1e, f). Incomplete alopecia, associated with follicular nodules most prevalent above the upper lip was seen. Hands and fingernails examination revealed hyperkeratosis and distal onycholysis.

**Case 2** A Caucasian male of 48 years presented erythematous and squamous lesions on the feet and toenails. A closer examination revealed scaling lesions on the inguinal area and buttocks, hands and fingernails plate hyperkeratosis and distal onycholysis.

**Case 3** A Caucasian female of 78 years reported a 2-week history of extensive erythema with papules and fine pustules appearing at the opening of hair follicles in the inguinal region (Fig. 2a, b). An intense erythema involved both buttocks and thighs (Fig. 2c). Examination of the left foot revealed sole and toenail/fingernail hyperkeratosis, with nail plate thickened, friable and yellowish (Fig. 2e, f). The left knee (Fig. 2d) and the right leg were also involved with flaking in net margins.

**Case 4** A Caucasian female of 69 years, with rheumatoid arthritis, treated for 20 years with therapeutic cycles of methotrexate (7.5 mg/week) and prednisone (5 mg/day), presented a chronic erythematous scaly dermatitis extended to the lower back and rear thigh area, diagnosed as psoriasis (Fig. 3e). Since 2006, she was treated with emollient cream and topical steroids without benefit. On physical examination, the patient revealed *tinea pedis* and *tinea unguium* with sole and toenails plate hyperkeratosis (Fig. 3a, b), squamous lesions on the elbow, on the back and left palm (Fig.3c, d, g). Involvement of the scalp with flaking dandruff and thinning hair was observed (Fig. 3f).

**Case 5** A Caucasian female of 68 years, with rheumatoid arthritis, treated for several years with prednisone (25 mg/day), presented a history of chronic erythematous scaly dermatitis diagnosed as psoriasis and treated with emollient cream without benefit. A closer examination revealed an intense lamellar desquamation of the toenails and fingernails, hyperkeratosis of the soles and the palms, scaling lesions with sharp margins in the breast, abdomen, inguinal area, buttocks and thighs, neck and chin.

Mycological analysis of all patient lesions was performed. Skin and nail samples were collected, examined under a light microscope (20 % KOH + 40 % DMSO preparation) and inoculated into Mycobiotic agar (Merck, KGAA, Germany) to detect dermatophytes. Molds identification was based on macroscopic and microscopic characters of the colonies after 15 days of incubation at 25 °C.

All patients had dermatophytosis and concurrent lesions caused by two variants of *T.rubrum*:

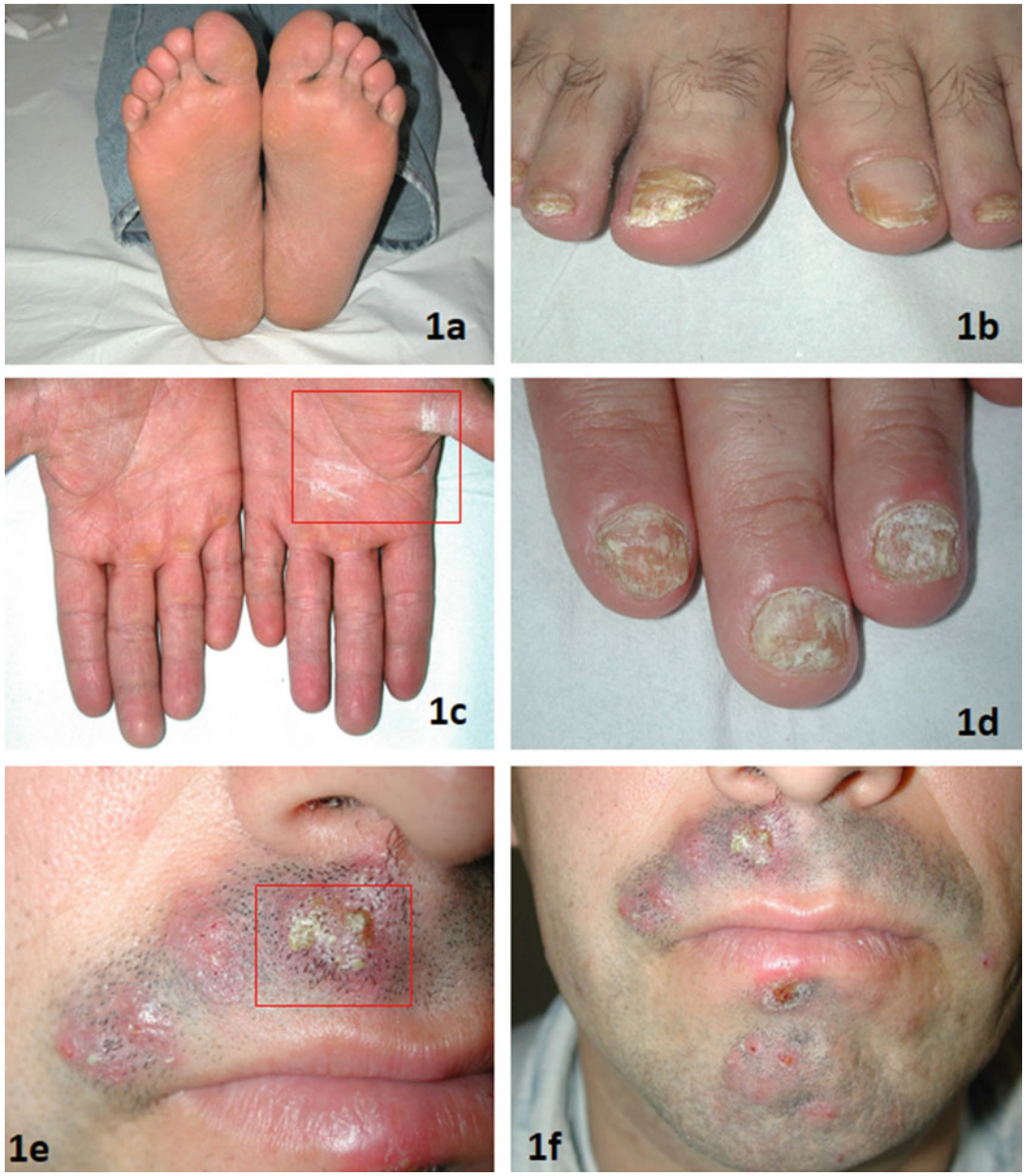


**Fig. 1** Case 1. A 42-year old, male, born in Ecuador. Squamous lesions on the soles, toenails, palms and nail plates (**a**–**d**); extensive erythema in the chin and beard with follicular nodules above the upper lip (**e**, **f**)

downy white-colored colonies with reverse pigment brownish-yellow (Cases 1, 2, and 3) or deep wine-red (Cases 4, and 5). Scant teardrop-shaped microconidia along septate hyphae were observed on microscopic colonies examination.

The primary lesion was localized always in the foot (*tinea pedis*), in agreement with other studies (Larruskain et al. 2005). Secondary lesions distributed in other sites were the main demand for medical consultation: in all five cases, the anatomical sites mainly interested were the inguinal area, buttocks, palms and fingernails (*tinea unguium*). In only one case, *tinea capitis* was observed (Case 4). Patient

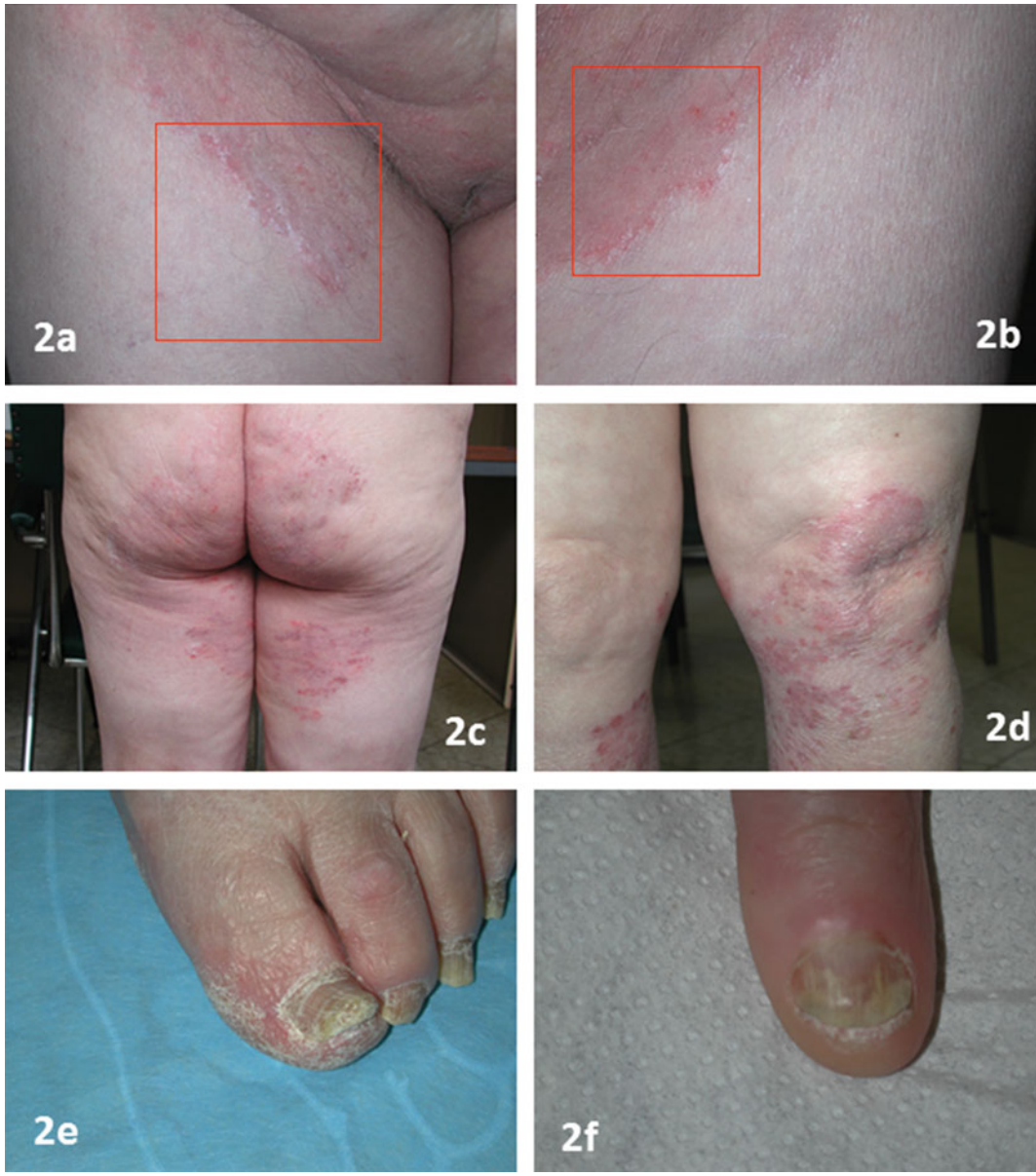


**Fig. 2** Case 3. A 78-year old, female, Caucasian. Extensive erythema with papules at the opening of hair follicles in the inguinal region (**a**, **b**), buttocks and thighs (**c**); left knee with flaking in net margins (**d**); toenail and fingernail hyperkeratosis (**e**, **f**)

4 under methotrexate therapy and patient 5, under corticosteroid therapy had risk factors predisposing them to fungal spread. *Tinea* in such cases tends to be chronic and extended, mimicking various skin diseases, such as psoriasis, eczema, etc., as in Patients 4 and 5 (Atzori et al. 2012; Tan et al. 2014).

For all patients a successful treatment with topical (azoles) and systemic (terbinafine hydrochloride 250 mg/day) antimycotics was carried out. In details, in patient 1, after 4 weeks of treatment, all skin lesions were completely healed and culture results were negative; both direct mycological and culture were negative

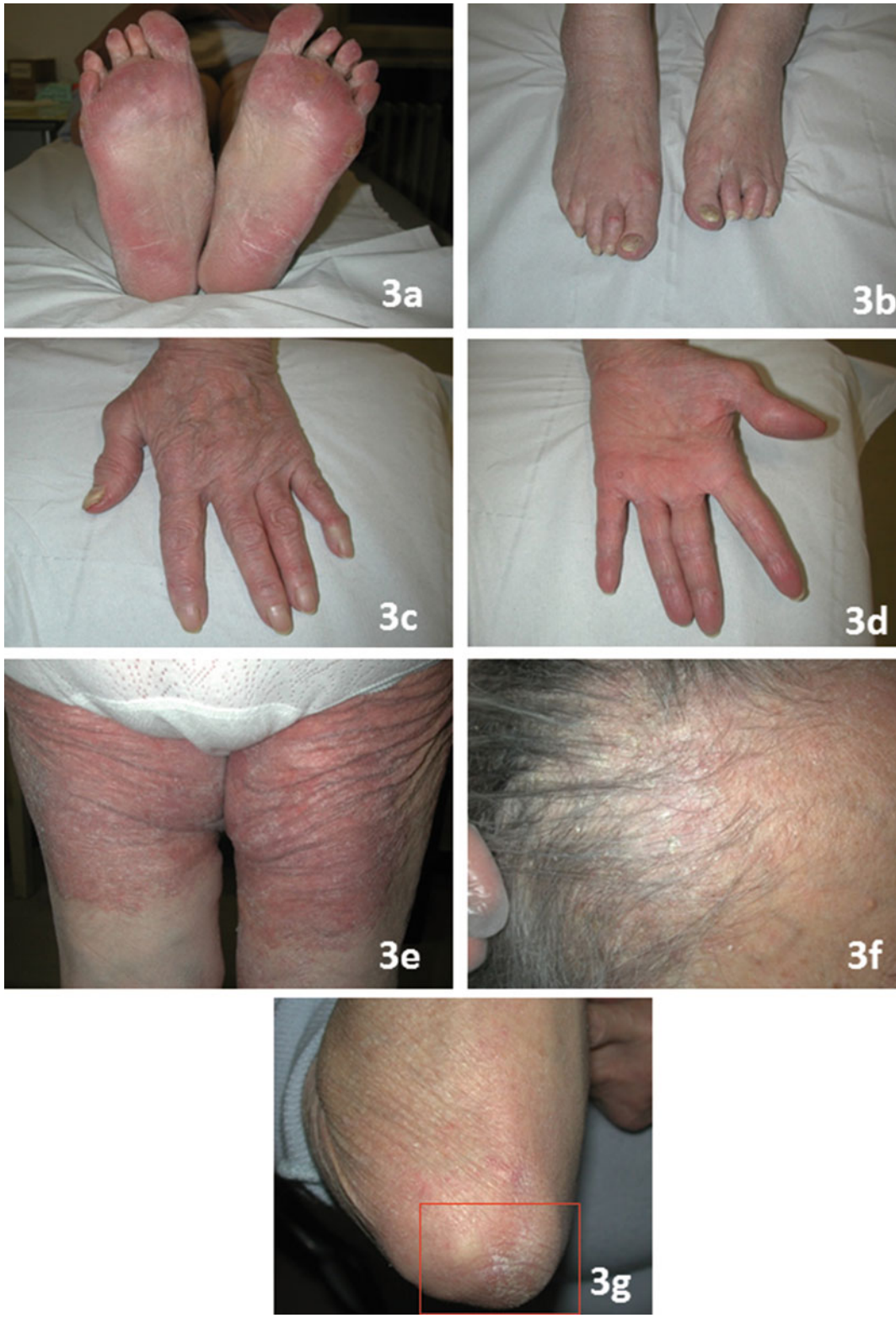


**Fig. 3** Case 4. A 69-year old, female, Caucasian, with rheumatoid arthritis. Sole and toenails hyperkeratosis (**a**, **b**); back and left palm squamous lesions (**c**, **d**); extensive erythema on lower back and rear thigh area diagnosed as psoriasis (**e**); scalp with flaking dandruff and thinning hair (**f**); squamous lesions on the elbow (**g**)

also for nails after 3 months. In patient 2, all lesions were completely healed and culture results were negative after 12-weeks of treatment. In patient 3, all skin lesions were completely healed after 6 weeks of treatment; both direct mycological and culture were negative for nails after 4 months. In patient 4, after 4-weeks of treatment, all skin lesions were completely healed; both direct mycological and culture were negative also for nails and scalp after 5 months. In patient 5, after 6-weeks of treatment, all skin lesions were completely healed and culture results were negative; the nail lesions were alleviated after 5-months therapy.

The five clinical cases reported in this study are considered dermatophytosis, affecting both immunocompetent and immunodeficient patients, and fulfilled the diagnostic criteria of *T.rubrum* chronic dermatophytosis, as indicated by the literature (Zaias and Rebell 1996; Böhmer and Korting 1999; Kick and Korting 2001; Balci and Cetin 2008; Piñeiro et al. 2010; Kong et al. 2015). Since in our group of patients from the beginning a correct therapeutic treatment was not carried out or misapplicated, a gradual spread of the infection occurred to the toenails, as secondary site involved, constituting the reservoir of infection that spread later to other sites, such as legs, groin, hands, face and scalp. On the other hand, it has to be underlined that *tinea unguium* is an infection usually more resistant to treatment, whose eradication is difficult even with appropriate therapy (Gupta and Cooper 2008).

For fungal infection eradication, diagnosis must be based on both a correct patient history and an adequate microbiological study that includes the identification of the species isolated. Therefore, it is essential a careful examination of the patient *in toto* to avoid inappropriate or wrong therapeutic treatment. In fact, as in the first patient, the antibiotic treatment was established solely on the observation of highly inflammatory facial injuries that did not present the typical clinical features of *T.rubrum* infection (Yin et al. 2011); hence, the treatment being wrong was ineffective.

In conclusion, our data indicate that in all cases of suspected syndrome or when skin involvement is extended to multiple sites, the localization of all lesions, the isolation and the identification of the causative fungus are essential to establish the diagnosis, prognosis and the setting of a correct antifungal therapy to avoid recurrences.

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# Antioxidant Hydroxytyrosol-Based Polyacrylate with Antimicrobial and Antiadhesive Activity Versus *Staphylococcus Epidermidis*

Fernanda Crisante, Vincenzo Taresco, Gianfranco Donelli, Claudia Vuotto, Andrea Martinelli, Lucio D'Ilario, Loris Pietrelli, Iolanda Francolini, and Antonella Piozzi

**Abstract**

The accumulation of reactive oxygen species (ROS) in microbial biofilms has been recently recognized to play a role in promoting antibiotic resistance in biofilm-growing bacteria. ROS are also over-produced when a medical device is implanted and they can promote device susceptibility to infection or aseptic loosening. High levels of ROS seem also to be responsible for the establishment of chronic wounds.

In this study, a novel antioxidant polyacrylate was synthesized and investigated in terms of antimicrobial and antibiofilm activity. The polymer possesses in side-chain hydroxytyrosol (HTy), that is a polyphenolic compound extracted from olive oil wastewaters.

The obtained 60 nm in size polymer nanoparticles showed good scavenging and antibacterial activity versus a strain of *Staphylococcus epidermidis*. Microbial adherence assays evidenced that the hydroxytyrosol-containing polymer was able to significantly reduce bacterial adhesion compared to the control. These findings open novel perspective for a successful use of this antioxidant polymer for the prevention or treatment of biofilm-based infections as those related to medical devices or chronic wounds.

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**Keywords**
Antioxidant polymers • Hydroxytyrosol • Medical device-related infections • Microbial biofilm • Nanoparticles

## 1 Introduction

Indwelling medical devices are of increasing importance in modern medical care and their use is continuously expanding. Infection is the main complication associated to medical device implantation. Medical device-related infections are supported by the presence onto the device surfaces of sessile bacterial communities known as biofilms (Habash and Reid 1999; Mack et al. 2006; Costerton et al. 2007; Francolini and Donelli 2010). These biofilm-based infections are usually polymicrobial (Wolcott et al. 2013) and exhibit increased antibiotic resistance (Hoiby et al. 2010; Mah 2012). Therefore, they represent a serious health risk in hospital settings (Al Mohajer and Darouiche 2012; Donelli and Vuotto 2014). The development of antimicrobial polymeric coatings is one of the most promising strategies to prevent medical device-related infections (Francolini and Donelli 2010; Campoccia et al. 2013; Francolini et al. 2015). In the last decades, different antimicrobial agent-releasing polymers have been developed by adsorption or impregnation with metal ions (Stickler et al. 1996; Francolini et al. 2010; Chernousova and Epple 2013; Wang et al. 2015), antiseptic agents (Rupp et al. 2005; Monzillo et al. 2012) and antibiotics either alone (Raad et al. 1998; Hanna et al. 2003; Piozzi et al. 2004; Ramos et al. 2011; Francolini et al. 2013) or in combination with antiseptics (Jamal et al. 2014) and biofilm dispersing agents (Donelli et al. 2007; Mansouri et al. 2013). More recently, antifouling polymers able to repel microbes have been also investigated (Siedenbiedel and Tiller 2012; Campoccia et al. 2013; Francolini et al. 2014a). Different materials, including metals, ceramics and polymers, are used for the manufacturing of medical devices, among which synthetic polymers are the best candidates thanks to their tuneable physico-chemical properties.

In order to find out novel strategies to address biofilm based-related infections, the phenomena occurring at the biomaterial/tissue interface should be considered. It is known that the host biological response to the implantation of a device (also called foreign body reaction) includes a cascade of cellular events including protein adsorption, adhesion and activation of immune cells, fibrosis and infection (Gristina 1994; Balasubramanian et al. 1999; Tang et al. 1999; Anderson et al. 2008). A consequence of the material-mediated inflammatory response is the over-production of reactive oxygen species (ROS), such as hydroperoxide, hypochloride, hydrogen peroxide and hydroxyl anions, released by activated phagocytes (Fialkow et al. 2007). This phenomenon contributes to the oxidative degradation of the device itself (Sutherland et al. 1993) and increase device susceptibility to aseptic loosening or infection (Gristina 1994). Furthermore, the accumulation of ROS in the microbial biofilm can promote antibiotic resistance in biofilm-growing bacteria as recently demonstrated in different biofilm communities (Boles and Singh 2008). Particularly, in patients with cystic fibrosis the oxidative stress caused by chronic lung inflammation was shown to be associated with the occurrence of antibiotic resistant bacteria in the lung (Ciofu et al. 2005). In addition, an increasing number of reports suggests that ROS participate in signaling pathways in bacteria and fungi regulating biofilm formation (Cap et al. 2012; Villa et al. 2012). Geier and colleagues (2008) showed that the addition of hydrogen peroxide to *Mycobacterium avium* cultures, as stimulus of the oxidative stress response, resulted in enhanced biofilm formation. Of particular relevance are the recent studies of Martins-Green and coworkers in which the role of high levels of ROS in establishment of chronic wounds was demonstrated (Dhall et al. 2014a, b).

Particularly, authors performed experiments in mice in which wounds were infected with biofilm-forming bacteria and the redox imbalance was enhanced by inhibiting the activity of antioxidant enzymes. They showed that wounds containing high levels of ROS remained open for several weeks, did not re-epithelialize and the granulation tissue lacked vascularization. Thus, excessive ROS production induced chronic inflammation in wounds and, combined with biofilm, created a toxic environment responsible for impaired wound healing Therefore, the use of antioxidants in combination of antimicrobial agents can be considered a novel promising strategy to prevent or treat biofilm-based infections.

Taking this as a clue, we planned the synthesis of a novel antioxidant polymer containing the antioxidant molecule in side-chain, to be investigated as antioxidant and antibiofilm agent for biofilm-based infections.

Many natural extracts rich in polyphenols have shown a combined antioxidant and antimicrobial activity (Visioli et al. 2002; Jung et al. 2012; Manea et al. 2014; Xu et al. 2014). In this work, hydroxytyrosol, a polyphenol extracted from olives, was chosen as antioxidant molecule since it possesses good antioxidant and antimicrobial activity (Visioli et al. 2002; de Pinedo et al. 2007), as well as anti-inflammatory (Visioli et al. 2002; Biesalski 2007) and antiplatelet aggregation properties (Correa et al. 2009). In particular, it has been demonstrated to be effective against a series of microorganisms including *Staphylococcus epidermidis, Staphylococcus aureus*, *Vibrio cholerae* and *Salmonella* species (Granados-Principal et al. 2010).

In the last decade, polyphenol polymeric conjugates have been proposed as innovative materials to be applied in several industrial fields ranging from materials science to biomedical, pharmaceuticals and cosmetics (Cirillo et al. 2014).

Although some studies have investigated the antioxidant activity of polymers functionalized with different natural antioxidant molecules (Boudreaux et al. 1996; Ortiz et al. 1999; Williams et al. 2009; Wattamwar et al. 2012), only few have been devoted to the investigation of the antimicrobial activity of these antioxidant polymers (Taresco et al. 2015a, b).

In our study, tyrosol (Ty), an abundant phenolic compound also present in olive oil, was used as a precursor molecule of HTy since this last compound is poorly available and expensive. An acrylic monomer containing Ty was synthesized and used for polymerization. Later, the cathecolic function was inserted by a chemo- and regio-selective reaction (Bernini et al. 2008).

After polymer physico-chemical characterization, the antioxidant, antimicrobial and antibiofilm activities of polymer were investigated. The polymer free radical scavenging activity was evaluated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a model radical. The antimicrobial activity was tested by using a *S. epidermidis* reference strain since this species is one of those mainly involved in medical device-related infections. The obtained results suggested as the newly synthesized polymer could be employed not only to suppress the oxidative stress but also to prevent medical device-related infections.

## 2 Materials and Methods

### 2.1 Materials

Sodium metabisulphite was purchased from Carlo Erba. Tyrosol (2-(4-Hydroxyphenyl)ethanol, Ty) was purchased from Wako Chemicals GmbH. Acrylic acid (AA), hydroquinone, potassium persulfate, sodium metabisulphite, p-toluenesulfonic acid (pTSOH), sodium dithionite, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), methanol and chloroform HPLC grade were purchased from Sigma Aldrich. All chemicals were of analytical grade and used as received. The reagent 2-iodoxybenzoic acid (IBX) was prepared in the laboratory as described in the literature (Frigerio et al. 1999). 2-(3′,4′-dihydroxyphenyl)ethanol (Hydroxytyrosol, HTy) was prepared in the laboratory as described in the literature (Bernini et al. 2008).

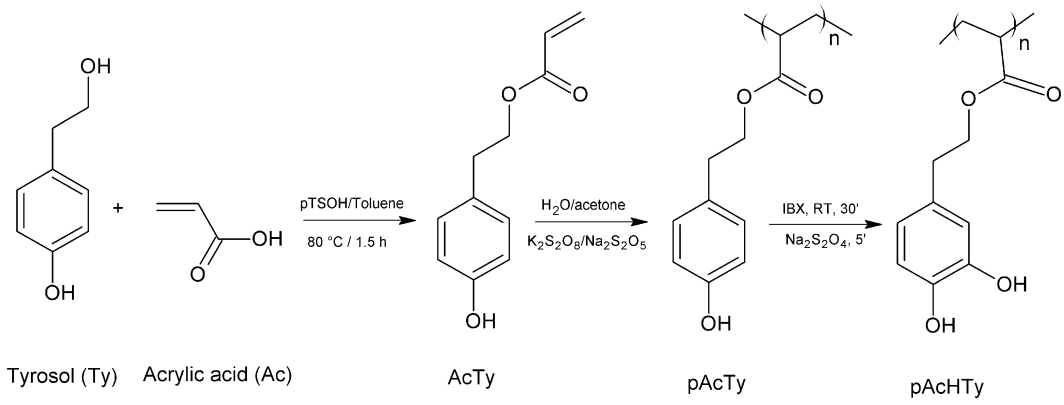


**Fig. 1** Synthesis of tyrosol-containing (pAcTy) and hydroxytyrosol containing (pAcHTy) polymers

## 2.2 Synthesis of Poly 2-(3,4-Hydroxyphenyl)Ethyl Acrylate (pAcHTy)

The hydroxytyrosol-containing polymer was obtained following the procedure reported in the Fig. 1. In the first step, an acrylic derivative of Tyrosol (AcTy) was obtained by Fischer esterification of acrylic acid (Ac) with Tyrosol (Fig. 1). Particularly, Ty (0.1 mol) was dissolved in toluene (75 mL) in presence of hydroquinone (20 mg) and *p*-toluenesulfonic acid (pTSOH, 750 mg). Then, Ac (0.12 mol) was added to the mixture and the reaction was refluxed for 1.5 h in a flask equipped with Dean-Stark apparatus. At the end of the reaction, the mixture was cooled, treated with sodium bicarbonate (6 g) and water (1.5 mL) and anhydrous sodium sulfate, filtered and dried under vacuum to remove toluene. The obtainment of AcTy was confirmed by $^1$H-NMR-analysis (300 MHz, $D_2O$): $\delta = 2.91$ (t, $J = 7.0$ Hz, 2H, C*H2*CH2O), 4.33 (t, $J = 7.0$ Hz, 2H, CH2C*H2*O), 5.83 (dd, $J1 = 10.5$ Hz, $J2 = 1.5$ Hz, 1H, C*H*H = CH), 6.12 (dd, $J1 = 10.5$ Hz, $J3 = 17.4$ Hz, 1H, CHH = C*H*), 6.39 (dd, $J3 = 17.4$ Hz, $J2 = 1.5$ Hz, 1H, CH*H* = CH), 6.78 (2H, d, J = 8.7, aromatic protons C*H* = CH), 7.09 (2H, d, J = 8.7, aromatic protons C*H* = CH). The reaction yield was >95 %.

AcTy (1 mmol) was dissolved in a water/acetone (1:1 w/w) mixture and submitted to radical polymerization (Fig. 1) by employing potassium persulfate (0.03 mmol) and sodium disulfite (0.1 mmol). After stirring for 50 min at room temperature, acetone was distilled off by heating at 60 °C and the reaction was kept at 80 °C for 2 h. A stable suspension of polymer (pAcTy) nanoparticles in water was obtained. The polymer pAcTy was recovered by centrifugation.

To obtain the hydroxytyrosol-containing polymer (pAcHTy), the pAcTy nanoparticles suspended in water were one-pot oxidized by 2-iodoxybenzoic acid (IBX, 1.2 mmol) and reduced by sodium dithionite (2 mmol) in order to covert tyrosol in hydroxytyrosol (Fig. 1). The pAcHTy nanoparticles were recovered by centrifugation at 3500 rpm for 15 min, washed with water to remove sulfur by-products and dried under vacuum at 25 °C for 1 day. Since pAcHTy was not soluble either in water or in common organic solvents, it was not possible to perform $^1$H-NMR analysis. Therefore, the oxidation yield was estimated by hydrolysis of the polymer side-chain by placing 200 mg of polymer in contact with 10 mL of 1 M NaOH at room temperature for 48 h. The solution was then acidified with 1 M HCl, extracted three times with ethyl acetate and washed first with saturated NaCl solution and then with water. The crude product was recovered and analyzed by $^1$H-NMR.

## 2.3 Characterization of Polymers

$^1$H-NMR analysis was performed by a Varian XL 300 in chloroform-d as a solvent. Elemental

analysis was carried out by a Carlo Erba Instrument EA 1110 CHNS-O elemental analyser.

Gel permeation chromatography (GPC) was carried out at 30 °C in HPLC grade chloroform at 1 mL/min by using a 150-C Waters GPC apparatus equipped with a differential refractive index detector. Monodisperse polystyrene samples with molecular weight ranging from $1.3 \times 10^3$ to $1.5 \times 10^6$ g/mol were used as standards. Two crosslinked polystyrene (PS) columns (Water Ultrastyragel) with a separation range $2 \times 10^3$ to $1 \times 10^6$ g/mol (linear) and 200 to $3 \times 10^4$ g/mol were used.

Differential scanning calorimetry (DSC) analysis was performed from −100 to +200 °C, at 10 °C/min, under $N_2$ by using a Mettler TA-3000 DSC apparatus. Thermogravimetric analysis (TGA) was carried out in the temperature range 30–500 °C at 10 °C/min under $N_2$ flow by employing a Mettler TG 50 thermobalance.

Size distribution of pAcTy and pAcHTy nanoparticles in water was assessed by a Laser Diffraction Particle Size Analyzer (LS 13320, Beckman Coulter), while their morphology was observed by scanning electron microscopy (SEM, LEO1450VP, Assing). For SEM observations, a suspension of polymer nanoparticles in THF was layered on a SEM stub and dried under vacuum at 30 °C. Then, the sample was gold coated and observed.

## 2.4 Evaluation of Antioxidant Activity

The antioxidant activity of pAcHTy was determined by using the DPPH method (Brandwilliams et al. 1995) and compared with that of hydroxytyrosol alone. For each compound, different concentrations (expressed as mol of antioxidant/mol DPPH) were tested.

To evaluate the antioxidant activity of hydroxytyrosol, different HTy amounts were dissolved in methanol (4 mL). Each of the so obtained solutions was added to 10 mL of a DPPH solution in methanol ($1.5 \times 10^{-4}$ mol/L). After 30 min at room temperature, the absorbance of the solutions at 520 nm was determined. Then, the amount of residual DPPH, evaluated from a calibration curve, was plotted as a function of the antioxidant/DPPH molar ratio, in order to extrapolate the Efficient Concentration ($EC_{50}$) defined as the mole of the antioxidant needed to decrease the initial DPPH concentration by 50 %.

To evaluate the antioxidant activity of pAcHTy, measurements were performed by using the same DPPH method with some modifications (Serpen et al. 2007) due to the poor polymer solubility. Particularly, different amounts of pAcHTy were suspended in 60 mL of methanol. Then, a 20 mL aliquot of a DPPH solution in methanol (0.5 mM) was added to each suspension. The vials were stirred for 30 min to facilitate the surface reaction between the insoluble polymer and the DPPH radical. Then, the polymer was recovered by centrifugation at 3500 rpm for 2 min and the absorbance of the supernatant was measured at 520 nm.

## 2.5 Evaluation of Antimicrobial Activity of Polymers

A reference strain of *S. epidermidis* (ATCC 35984) was employed in the experiments. The disk diffusion test was performed by embedding cellulose disks with a THF solution of each compound. In the case of pAcHTy, a suspension of the polymer in water was prepared given its poor solubility in common solvents. For each sample, three concentrations (1, 5 and 10 mg/mL) were used. The embedded cellulose disks were placed onto Tryptic soy agar (TSA) Petri plates previously seeded with $10^8$ CFU/mL of *S. epidermidis strain*. Following incubation at 37 °C for 24 h, the diameters of inhibition zones of bacterial growth around the disks were measured.

The minimum polymer concentration able to inhibit bacterial growth, taken as the MIC, was determined by turbidimetric assay (Francolini et al. 2014b). Different amounts of each compound (0.5, 1, 2.5, 5, 7.5, 10, 15 and 20 mg) were

placed into test tubes containing 2 ml of TSB. Each test tube was inoculated with 20 $\mu$L of *S. epidermidis* suspension 0.5 McFarland (0.125 OD at 550 nm). A tube containing the bacterial suspension without polymer was used as control. Following incubation for 24 h at 37 °C, the polymer was separated by centrifugation and the bacterial suspension was harvested. The antimicrobial effect was assessed by measuring the absorbance of the bacterial suspension at 550 nm. The minimum polymer concentration that inhibited the bacterial growth (no variation of the absorbance of the test tube before and after incubation) was taken as the MIC.

### 2.6 Assessment of pAcHTy Antibiofilm Activity

The ability of pAcHTy to control bacterial adhesion was evaluated by cell-count determining the number of CFUs adhered per polymer surface unit after overnight incubation of the polymer with a bacterial suspension. Particularly, a thin polymer film was prepared by dispersing polymer nanoparticles in THF and layering the resulting suspension on teflon plates. Following solvent evaporation, a film (ca. 50 μm thickness) constituted by polymer nanoparticles attached together was obtained. The polymer film (surface area ca. 3 $cm^2$) was immersed in 2.5 ml of bacterial suspension ($10^8$ $CFU_s$/ml, 0.125 OD at 550 nm) in MH and incubated for 24 h at 37 °C. A microscope slide coverslip (10 mm diameter) was used as a negative control. Following incubation, the polymer film and the coverslip were collected and the bacterial suspensions were harvested. The samples were washed twice with phosphate buffer (PBS, pH = 7.4) to remove loosely adherent bacteria, placed into tubes containing 10 mL of PBS and sonicated for 1 min to detach adherent bacteria. Five 10-fold dilutions were prepared, and three 10 μl aliquots of each dilution were placed on TSA plates. Colony-forming units were counted after 18 h incubation at 37 °C in the first dilution in which colonies were well separated. Finally, considering the dilution factor and the surface area of the samples, the number of CFUs per surface unit (CFUs/$cm^2$) was calculated.

To evaluate a possible antimicrobial effect of the polymer film on the surrounding environment, after incubation with the polymer the absorbance of the bacterial suspension was measured at 550 nm and compared with that of the control. The bacterial growth inhibition (BGI) was determined as follows:

$$BGI\,(\%) = \left(1 - \frac{A_{test\ tube} - A_0}{A_{control} - A_0}\right) \times 100$$

where $A_{test\ tube}$ and $A_{control}$ are the absorbances of the test and the control tube after 24 h-incubation, and $A_0$ is the absorbance of the bacterial suspension before incubation.

To evaluate the durability of the antimicrobial effect of the polymer, the test was repeated daily by transferring the polymer film into a tube containing a freshly prepared bacterial inoculum, until the bacterial growth inhibition was no longer noticed (BGI ≅ 0).

## 3 Results and Discussion

In this study, a polymer bearing hydroxytyrosol in side-chain was developed and tested in terms of antioxidant, antimicrobial and antiadhesive activity. First of all, an acrylic monomer containing tyrosol (AcTy) was synthesized. Then, this monomer was submitted to radical polymerization to obtain a polymer having tyrosol in side-chain (pAcTy). After polymerization a stable suspension of pAcTy nanoparticles in water was obtained. The confirmation of AcTy polymerization was given by $^1$H-NMR. Indeed, in the spectrum of the polymer the signals at 5–6 ppm related to the protons of the double bond of the acrylic moiety were absent (spectrum not reported), suggesting a complete monomer conversion (reaction yield >95 %). The polymer pAcTy resulted to be soluble in some organic solvents such as tetrahydrofuran, chloroform and toluene.

Later, pAcTy was oxidized and reduced to convert the side-chain tyrosol in hydroxytyrosol.

**Table 1** Elemental analysis of pAcTy and pAcHTy

| | C (%) | | H (%) | | |
|---|---|---|---|---|---|
| Sample | Theoretical | Experimental | Theoretical | Experimental | S (%) |
| pAcTy | 69 | 68 | 6 | 6 | – |
| pAcHTy | 63 | 64 | 6 | 7 | – |

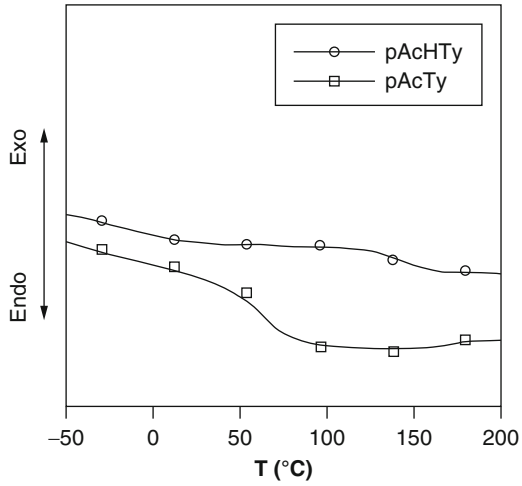


**Fig. 2** DSC curves of the pAcTy and pAcHTy

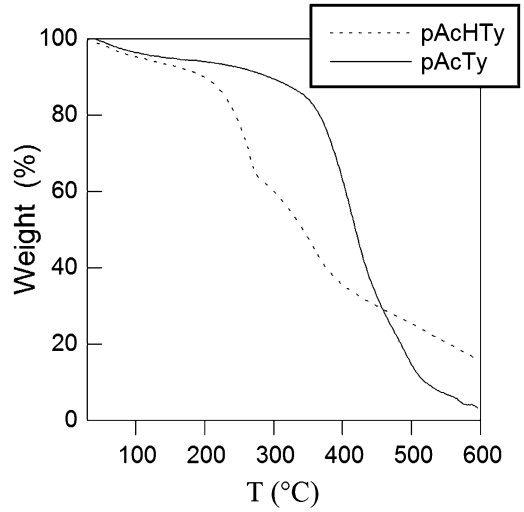


**Fig. 3** TGA curves of the pAcTy and pAcHTy

After reaction and purification, a suspension of pAcHTy nanoparticles was obtained (yield in weight ca. 80 %). These nanoparticles resulted to be insoluble in common solvents presumably due to cross-linking of the quinones (Yu et al. 1999). Therefore, to determine the HTy content in pAcHTy, hydrolysis of the polymer side-chain was carried out under conditions described in Materials and Methods. Results showed a HTy content of 60 % with respect the initial Ty. This oxidation yield could be underrated due to a possible incomplete polymer side-chain hydrolysis. Elemental analysis confirmed the composition of the polymer repeat unit and the efficiency of the purification given the absence of sulfur by-products (Table 1).

Due to pAcHTy insolubility, GPC analysis was performed only on the soluble polymer pAcTy. Results showed a molecular weight of ca. 5000 g/mol. We can assume a similar molecular weight for pAcHTy since the experimental conditions adopted for pAcTy oxidation were not degradative for the polymer main-chain.

DSC analysis evidenced that both the synthesized polymers were mainly amorphous showing a glass transition at approximately 60 °C for pAcTy and 135 °C for pAcHTy (Fig. 2). The higher glass transition temperature of pAcHTy is in agreement with the cross-linking hypothesis (Stutz et al. 1990). To verify polymer thermal stability, thermogravimetric analysis was carried out. The thermal profiles (Fig. 3) showed less stability of pAcHTy than pAcTy. The presence in the pAcHTy thermogram of two decompositions, at ca. 230 °C and 330 °C, can be attributed to the coexistence of two type of side residues, tyrosol and hydroxytyrosol, that did not allow a good packing of the polymer chains.

The mean particle size of pAcTy and pAcHTy in water suspension was about 60 ± 5 nm, measured by Laser Diffraction Analyzer. After drying under vacuum, nanoparticles were found to form aggregates of ca. 600 nm in size, as shown in the SEM micrograph (Fig. 4).

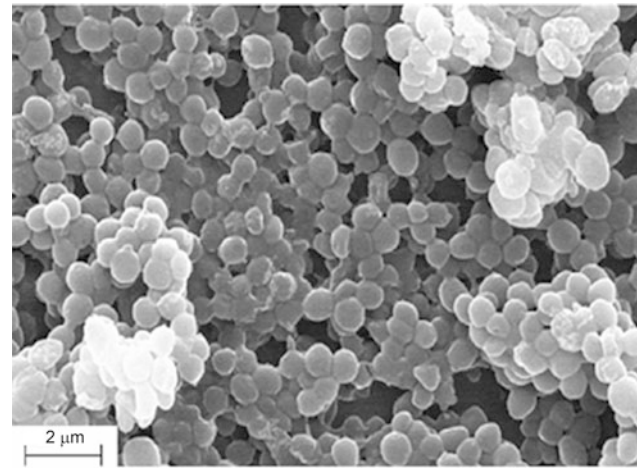


**Fig. 4** SEM micrograph of pAcHTy nanoparticles, obtained by evaporation of pAcHTy suspension in THF

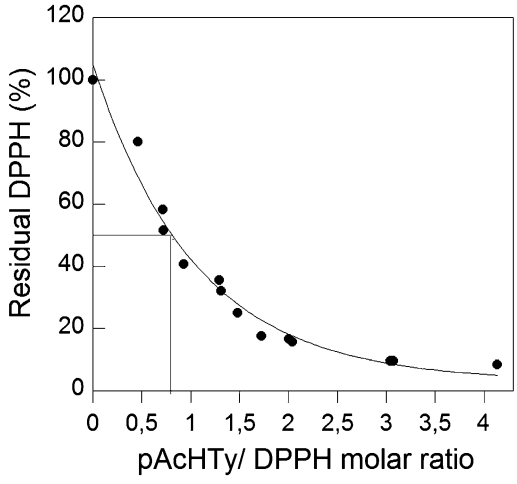


**Fig. 5** Antioxidant activity of pAcHTy measured by DPPH radical. The residual DPPH percentage was reported as a function of pAcHTy/DPPH molar ratio. The EC50 value was estimated in correspondence of the 50 % residual DPPH

The antioxidant activity of HTy and pAcHTy was determined by using DPPH as a free anionic radical. In the DPPH method, the reaction degree depends on the hydrogen-donating ability of the antioxidant species. In Fig. 5, the residual DPPH percentage as a function of pAcHTy/DPPH molar ratio is reported. From this graph, a $EC_{50}$ value of $0.80 \pm 0.02$ mmol pAcHTy/mmol DPPH was estimated. The $EC_{50}$ of hydroxytyrosol was instead found to be $0.18 \pm 0.02$ mmol HTy/mmol DPPH. The pAcHTy $EC_{50}$ is lower than that of hydroxytyrosol due to the higher molecular weight and insolubility of the polymer. However, the antioxidant activity showed by pAcHTy is rather significant compared to that found in the literature for other modified natural or synthetic polymers (Curcio et al. 2009; Iemma et al. 2010). The tyrosol-containing polymer pAcTy did not show antioxidant activity.

The antimicrobial activity of samples was evaluated by the disk diffusion test and turbidimetric analysis. As for the disk diffusion test, Ty and the AcTy monomer did not show any inhibition zone due to their poor solubility in water. On the contrary, HTy showed an inhibition zone of ca. 10 mm at 10 mg/mL thanks to the higher hydrophilicity of the catecholic moiety with respect to the phenolic one. As expected, both pAcTy and pAcHTy did not show any inhibition zone of bacterial growth due to their high molecular weight and low solubility which hindered diffusion in agar. When the antimicrobial activity was assayed in broth, pAcHTy showed a good antimicrobial activity with a MIC value of 2.5 mg/ml (Table 1). Conversely, the pAcTy showed a very high MIC (15 mg/ml) and therefore can be considered not active against the *S. epidermidis* tested strain. Besides, tyrosol resulted to be not active against the tested strain. These results are in agreement with the higher activity of hydroxytyrosol compared to tyrosol (Tuck and Hayball 2002).

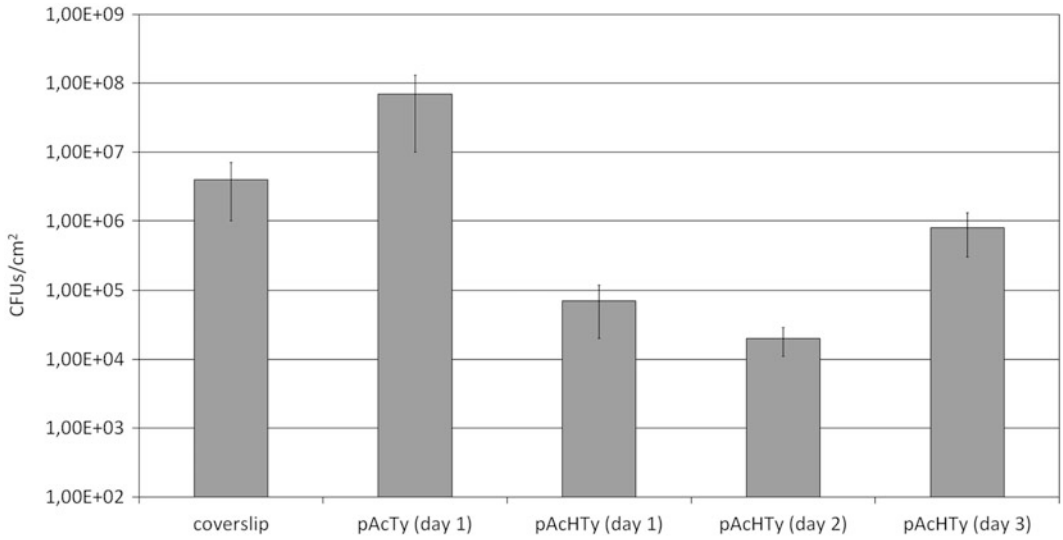


**Fig. 6** Adhesion of *S. epidermidis* (CFUs/cm$^2$) onto pAcTy at 1 day of incubation and pAcHTy at different incubation times. A coverslip was used as the negative control

To evaluate a possible activity of pAcHTy in preventing microbial adhesion, a pAcHTy film consisting of adhered nanoparticles was prepared and incubated overnight with a *S. epidermidis* suspension. After incubation, the number of CFU adherent per polymer surface unit was determined by count of the bacterial cells detached from the film while the bacterial growth inhibition was determined by measuring the absorbance of the bacterial suspension. The test was repeated daily to evaluate the durability of the antimicrobial effect.

In Fig. 6, the number of CFU/cm$^2$ of pAcTy at 1 day of incubation and pAcHTy at day 1, day 2 and day 3 of incubation is reported. A coverslip was used as negative control. As it can be observed, bacterial adhesiveness on pAcHTy was significantly reduced compared to both the control and pAcTy. Indeed, after 1 day of incubation, pAcHTy showed ca. 3-log reduction in bacterial adhesion compared to pAcTy. Surprisingly, the pAcHTy film was also able to inhibit the growth of the surrounding bacteria (Fig. 7a). This finding was presumably related to the release of pAcHTy nanoparticles from the polymer film. To confirm this hypothesis, the eluates showed antimicrobial activity. To verify the durability of the bacterial growth inhibition, the polymer film was transferring daily into a tube containing a freshly prepared bacterial inoculum, until the bacterial growth inhibition was no longer noticed. As shown in Fig. 7b, the bacterial growth inhibition was almost complete up to 2 days of incubation, was ca. 30 % at day 3 and negligible at day 4 and 5 (Fig. 7b). When the bacterial growth inhibition was almost complete (day 1 and day 2 of incubation) also the bacterial adhesion on the polymer film was significantly reduced (Fig. 6).

Overall, the obtained results are encouraging for a success of our polymer for biofilm prevention or treatment. Indeed, pAcHTy nanoparticles could be layered on the surface of medical device to prevent biofilm formation and infection development. Although further investigation will address this issue, we believe that the catecholic moiety of hydroxytyrosol will guarantee a good adhesiveness of the polymer to different materials including polymers and metals. Besides, we can hypothesize the use of pAcHTy nanoparticles to treat biofilm-based infections especially those related to chronic wounds which are characterized by elevated levels of ROS. pAcHTy nanoparticles could be used as topical agents to be applied to infected chronic wounds, alone or in combination with proper antimicrobial wound dressings, to treat inflammation and promote wound healing.

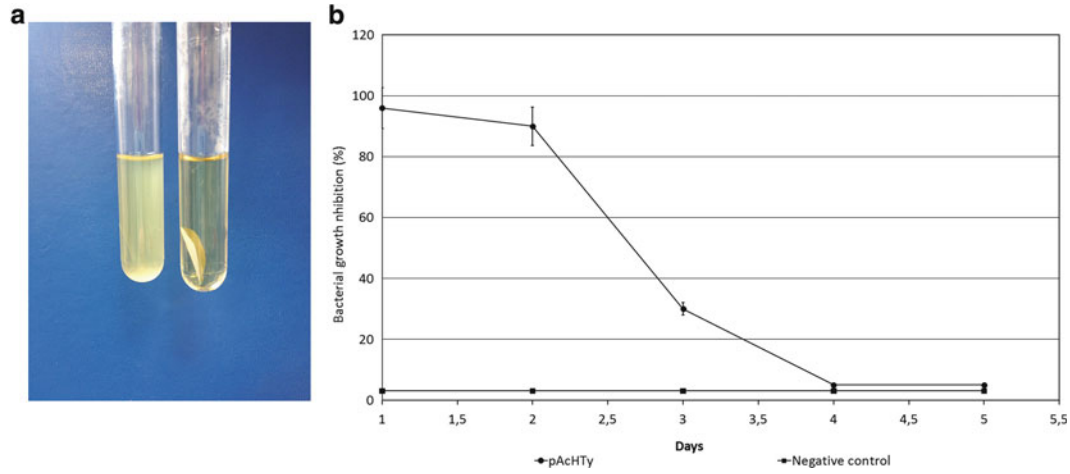


**Fig. 7** Image showing the test tubes containing a coverslip (*left*) and pAcHTy (*right*) in contact with the bacterial suspension after 1 day of incubation (**a**); bacterial growth inhibition at increasing incubation times (from day 1 to day 5) (**b**)

## 4 Conclusions

A novel antioxidant polyacrylate based on the natural polyphenol hydroxytyrosol was synthesized. For polymer synthesis, tyrosol was used as a hydroxytyrosol precursor since it is available at low cost. Free radical polymerization of an acrylic monomer containing tyrosol lead to the obtainment of 60 nm-in size nanoparticles that were further submitted to oxidation to convert tyrosol to hydroxytyrosol. The resulting pAcHTy possesses strong antioxidant activity ($0.80 \pm 0.02$ mmol pAcHTy/mmol DPPH) and good antimicrobial properties against *S. epidermidis* (MIC = 2.5 mg/mL). Microbial adherence assays showed as pAcHTy was able to cause a 2-log reduction in bacterial adhesion compared to the control. The developed antioxidant nanoparticles could be used as coatings for medical devices to prevent biofilm formation or as topical agents for chronic wounds to reduce inflammation and promote biofilm eradication.

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# The Role of Human Herpesvirus 8 in Diabetes Mellitus Type 2: State of the Art and a Medical Hypothesis

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**Abstract**

Diabetes is a common chronic disease due to an altered glucose metabolism, caused by the quantitative and/or qualitative dysfunction of the insulin hormone. Two types of diabetes are recognized: juvenile diabetes, or type 1, which has an autoimmune origin, and adult diabetes, or type 2 (DMT2), which covers 90–95 % of all diabetic patients.

The causes of DMT2 are not yet clear: heredity, life style, nutrition, and environment are considered the main risk factors. Several viral infections, namely cytomegalovirus, coxsackie and other enteroviruses, rubella and hepatitis C virus, have been claimed to be associated with some forms of diabetes. The direct role of viruses as a cause or as a risk of type 1 diabetes has been amply described in several recent reviews. Therefore, this review focuses attention on the role of a human herpes pathogenic virus in the onset of DMT2. By carrying out an analysis of recent literature, we describe the findings reported on an extremely deceitful virus, such as Human Herpes virus 8, and present a medical hypothesis on a possible relationship between this virus and DMT2.

**Keywords**

Human Herpesvirus 8 • Diabetes type 2 • Latent virus infection • Insulin resistance

## 1 Introduction

Diabetes is a very common chronic disease due to an altered glucose metabolism caused by the quantitative and qualitative dysfunction of the insulin hormone (Winter and Signorino 2002). Two types of diabetes are recognized: type 1, or

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juvenile diabetes, which has an autoimmune origin, and type 2, or adult diabetes (DMT2), which covers 90–95 % of all diabetic patients and is considered an increasing social disease in developing countries (Diabete – EpiCentro 2015). In Europe alone, there are more than 30 million clinically recognized type 2 diabetics, who need medical assistance (Diabete – EpiCentro 2015).

Persons with DMT2 produce insulin, but this is unable to find the right sensibility in the peripheral cells, which, as a consequence, are no longer able to uptake and metabolize glucose correctly. The causes of diabetes are not yet clear: heredity, life style, nutrition, obesity, and environment are considered the main risk factors. Several viral infections, namely cytomegalovirus, coxsackie virus, and rubella, have been claimed to be associated with some forms of diabetes (van der Werf et al. 2007; Jaïdane et al. 2010; Spagnuolo et al. 2013; Hober and Alidjinou 2013). Also chronic Hepatitis C virus (HCV) infection has been associated with an increased risk of developing insulin resistance (IR) and DMT2. Pre-existing HCV infection may increase the incidence of DMT2 in persons with known risk factors (obesity, family history, physical inactivity, hypertension, age). However, only a small percentage of DMT2 subjects can be chronically infected by HCV. In addition, another consideration is that DMT2 is a common complication of most liver diseases, independently of their etiology, especially at the advanced stage. In conclusion, HCV and DMT2 association indicates that HCV interferes directly through one or more of its proteins and/or indirectly by modulating the production of specific cytokines, such as TNF-α, with glucose metabolism (Negro and Alaei 2009; Pattullo and Heathcote 2010; Fallahi et al. 2013). The role of viruses as a cause or as a risk of type 1 and type 2 diabetes has been amply described in several recent reviews (van der Werf et al. 2007; Jaïdane et al. 2010; Spagnuolo et al. 2013; Hober and Alidjinou 2013; Negro and Alaei 2009; Pattullo and Heathcote 2010; Fallahi et al. 2013), but, to date, no infectious agent has really been considered as the cause of DMT2. Therefore, this paper focuses attention on the possible role of the last discovered human pathogenic herpes virus in the onset of DMT2. In this review we carry out an analysis of the recent literature and describe the findings reported on an extremely deceitful virus, namely Human Herpes virus 8 (HHV8).

## 2 Human Herpes Virus 8 and Diabetes Mellitus Type 2

Some interesting studies have recently suggested a possible association between DMT2 and HHV8. The virus has been found in more than 50 % of Sardinian diabetic subjects and in a high percentage of sub-Saharan persons with DMT2. Ingianni and Piras from our Department were the first to describe a possible association of DMT2 with latent infection by HHV8, on the basis of their epidemiological studies of DMT2 patients from the island of Sardinia, where diabetes seems to be highly frequent (Ingianni et al. 2007; Piras et al. 2014). They carried out studies on more than 400 persons with DMT2 and about 200 healthy blood donors. The HHV8 virus was detected by a PCR test on peripheral leucocyte DNA. The results indicated that more than 50 % (range 49–56 %) of DMT2 subjects were positive for HHV8 DNA, against a prevalence of about 12–15 % in the healthy controls ($p < 0.01$). The percentage rose to about 64 % when only the obese DMT2 subjects were considered (personal communication). The authors concluded that more than half of the DMT2 subjects in Sardinia are stably infected by HHV8 in a latent phase.

Subsequently, in 2008, Gautier's group (Sobngwi et al. 2008) published an elegant paper where they demonstrated that there is a strong relationship between HHV8 infection and a particular type of DMT2, which is characterized by a high level of ketosis. HHV8 infection in the sub-Saharan regions is known to be endemic and widely diffused. All participants in this study were black and of African origin: 187 were consecutive diabetic patients of whom 81 had ketosis-prone DMT2 and 106 had non-ketotic DMT2, while 90 individuals were non-diabetic control participants, who were

matched for age and sex. Anti-HHV8 antibodies were detected by immune-fluorescence methods against both latent and lytic HHV8 antigens. Furthermore, HHV8 DNA was also investigated in 22 of the participants at the clinical onset of diabetes. HHV8 antibodies were found in 71 patients (87.7 %) with ketosis-prone DMT2 vs. 16 patients (15.1 %) with non-ketotic DMT2, while in non-diabetic controls, anti-HHV8 antibodies were detected in about 40 % of subjects. HHV8 in genomic DNA was present in 6 of the 13 patients with ketosis-prone DMT2 tested at acute onset. The authors also claimed that HHV8 was able to infect human pancreatic β cells in culture *in vitro* and that HHV8 proteins were present in human islet cells, which had been cultured for 4 days in the presence of HHV8. The authors concluded that the presence of HHV8 antibodies was associated with ketosis-prone DMT2 in subjects of sub-Saharan African origin and that there was a direct activity of viral infection on the insulin-producing pancreas β cells.

HHV8 has a characteristic tropism for B lymphocytes and is known to be the causative agent of Kaposi sarcoma (KS), as well as of some B cell lympho-proliferative diseases, namely primary effusion lymphoma (PEL) and multicentric Castleman disease (reviewed by Ganem (2010) and Wen and Damania (2010)). Moreover, HHV8 exerts an important role in the functional regulation of infected cells: the infected cells become more resistant to toxic substances, radiation, chemical and physical stress and are able to have a prolonged lifespan as compared to non-infected ones (in fact they can be transformed into neoplastic cells, such as in Kaposi sarcoma and in lympho-proliferative diseases).

HHV8 infection of endothelial cells *in vitro* causes dramatic changes in the cellular phenotype which resembles the spindle shape of KS lesion cells (Ablashi et al. 2002). On the other hand, the effects of lytic and latent HHV8 infection on endothelial cell functions and the triggering of inflammatory processes are still largely unknown (Caselli et al. 2007; Gregory et al. 2012).

HHV8 infection induces profound modifications in the behavior of both primary and immortalized endothelial cells. In addition, HHV8 causes an intense transcriptional reprogramming in human endothelial cells (HUVEC) (Wang et al. 2004); stimulates the Warburg effect (cells produce energy mainly by a high rate of glycolysis followed by lactic acid fermentation) in latently infected TIME cells (immortalized human microvascular endothelial cells), with an increase in glycolysis and glucose consumption (Delgado et al. 2010), and activates hypoxia-induced factors (Carroll et al. 2006).

In a recent work, Rose et al. (2007) found that in HHV8-infected dermal micro-vascular cells (E-DMVEC), the expression of the insulin receptor (IRec) was strongly induced in latently infected cells. The binding of ligands to the IRec triggered a signal cascade that regulated cell growth and survival (Ottensmeyer et al. 2000; Raggo et al. 2005; Mcallister and Moses 2007). Moreover, over-expression of the IRec in KS tissue compared to normal skin was also reported by Wang et al. (2004).

The observation that HHV8 infection induces a strong enhancement of both insulin and glucose uptake in primary endothelial cells was also confirmed and extended by our group (Ingianni et al. 2013). The increase in insulin uptake is already evident in the lytic phase of the viral infectious cycle and reaches a maximum during the latent phase, whilst glucose uptake is slightly depressed during the lytic viral infection, but significantly enhanced compared with the control during the latent phase of viral infection. We concluded that HHV8 induced a marked and significant enhancement of both insulin and glucose uptake in infected HUVEC cells during the latent phase of viral infection.

HHV8 is believed to establish persistent infection for the duration of the host's existence, with a limited gene expression programme, which maintains the circularized viral DNA genome during cellular replication, with only occasional switching to lytic phase infection (Parravicini et al. 2000; Douglas et al. 2010). In addition, Watanabe et al. (2003) and Wang & Damania (2008) found that the HHV8 latency-associated nuclear antigen (LANA) prolonged the life span of primary HUVEC cells and augmented cell survival in the presence of apoptotic

inducers and under conditions of serum deprivation. Many other works have stressed that HHV8 infection induces intense and durable changes in the physiological properties of HHV8-infected cells (Ganem 2010; Carroll et al. 2006; Dourmishev et al. 2003; Guilluy et al. 2011), but very little is known about the metabolic modifications underlying these altered cell behaviors.

## 3 HHV8 Infection and Cell Metabolism

The metabolic profiles of HHV8-infected PEL cells are significantly different from that of primary B cells (Bhatt et al. 2012). Compared with the latter, both aerobic glycolysis and fatty acid synthesis (FAS) are up-regulated in PEL. Findings have also shown that aerobic glycolysis and FAS occur in a PI3K-dependent manner and appear to be interdependent. PEL over-express the fatty acid synthesizing enzyme complex and PEL is much more sensitive to a FAS inhibitor than primary B cells. Delgado et al. (2012) also found that virally infected cells, as well as cancer cells, have dramatically altered metabolic requirements. The group analyzed global metabolic changes induced by latent infection with HHV8 virus and Kaposi sarcoma, which is the most common tumor in AIDS patients. Following latent infection of endothelial cells by HHV8, approximately one-third of the nearly 200 measured cell metabolites were altered, including many metabolites of the anabolic pathways common to most cancer cells. The HHV8 induced pathways which are commonly altered in cancer cells include glycolysis, the pentose phosphate pathway, amino acid production, and fatty acid synthesis. In addition, several of the detectable long chain fatty acids detected in the screen were significantly increased by latent HHV8 infection. HHV8 infection leads to the elevation of the metabolites involved in the synthesis of fatty acids, and also to increased lipid droplet organelle formation in the infected cells. Fatty acid synthesis is required for the survival of latently infected endothelial cells, since the inhibition of key enzymes in this pathway leads to the apoptosis of infected cells. Metabolomic analysis provided insight as to how HHV8 virus can induce metabolic alterations, which give strong survival advantages to the infected cells.

The cell transforming ability of HHV8 has been associated with the activation of NF-kB, a nuclear factor playing a key role in promoting inflammation and cell proliferation; however, little is known about NF-kB activation during acute HHV8 infection. Caselli et al. (2007) used an established *in vitro* model of HHV8 acute productive infection in endothelial cells to investigate the effect of HHV8 on NF-kB activity and function. HHV8 rapidly and potently induced NF-kB activity in endothelial cells via stimulation of the IKB-kinase (IKK). Following IKK activation, HHV8 selectively triggered the production of high levels of monocyte chemoattractant protein 1 (MCP-1), whereas it did not affect the expression of other NF-kB–dependent pro-inflammatory proteins, including TNF-, IL-8, and RANTES. Deletion of the NF-kB–binding sites in the MCP-1 enhancer resulted in significant inhibition of HHV8 induced transcription. These results suggest that HHV8–induced MCP-1 may play an important role in promoting the inflammation and pathogenic angiogenesis typical of HHV8–associated lesions.

DMT2 is often associated with a general metabolic syndrome, which appears to have some kind of similar development and progression. Moreover, DMT2 is characterized by the presence of high concentrations of the acute phase proteins involved in innate immunity. These proteins are known to be able to induce insulin resistance, hypercholesterolemia, hypertension, and micro-albuminuria. HHV8 is able to permanently infect B lymphocytes in a latent state, inducing a chronic inflammatory response and also activating the production of a homolog of cell interleukin 6 (vIL-6), which can activate production of the acute phase inflammatory proteins (Seo et al. 2009). Subsequently, Gregory et al. (2012) studied viral and cytokine gene expression in the HHV8-THP-1 cell model

(a monocytic cell line) compared to that found in uninfected THP-1 cells. They discovered that several genes involved in the host immune response were down-regulated during latent infection, including genes for CD80 and CD86, as well as the cytokine TNF-α and interleukin-1β (IL-1β). They concluded that HHV8 is able to suppress some key immune response factors, enabling evasion from host detection. Another interesting finding regarding HHV8-cell interaction was reported by Bottero et al. (2013), who demonstrated that HHV8 infection induced reactive oxygen substances (ROS) at an extremely early stage in infection to facilitate its efficient entry into HMVEC-d cells via macropinocytosis. It is important to note that ROS can have a fundamental role in metabolic modifications, which can lead to the onset of DMT2, as will be discussed in a following paragraph.

In addition, HHV8 was found to disregulate both cholesterol and triglyceride metabolism (TG) *in vitro* (Angius et al. 2015). This work, performed in our Department, also studied lipid synthesis and metabolism in HHV8 infected HUVEC cells. Cholesterol and triglyceride synthesis were analyzed during both lytic and latent HHV8 infection for more than 3 weeks. A quantitative analysis of neutral lipids in infected cells was performed *in situ*. The results showed that, during the lytic phase, a depression of cholesterol and cholesterol ester synthesis could be observed, while triglycerides appeared to be enhanced as compared to controls. However, during the latent phase of infection, there was an increased synthesis of cholesterol and cholesterol esters in infected cells, whereas triglycerides were progressively lowered. Lipid synthesis inhibitors were used to examine the importance of lipid synthesis modification and neutral lipid accumulation in the pathogenesis of KS and neo-angiogenesis. An increase of TG-enriched lipids during the lytic phase of viral infection has often been reported for other viruses such as HCV. In fact, it has been suggested that the virus uses TG-enriched lipid droplets as a platform for the assembly of nascent virions, and that the HCV core protein is a main player in the manipulation of these organelles. Overall, these observations highlight the fact that a range of viruses (HBV, GBV-B, Dengue virus) have evolved mechanisms to interact with lipid droplets and possibly to subvert the function of these organelles, so as to use them as a platform for viral particle assembly (Angius et al. 2015).

We concluded that HHV8 infection impairs both triglyceride and cholesterol metabolism, with an up-regulation of triglyceride synthesis during the lytic phase and an up-regulation of cholesterol esters during the latent one.

## 4 Is HHV8 Infection a Risk Factor for the Onset of DMT2?

Obesity is generally recognized as being a risk factor for DMT2, although the mechanisms that lead to DMT2 in obese subjects are not clear. It is generally known that obesity can cause endoplasmic reticulum stress and a consequent unfolded protein response (UPR), which can be the cause of a reduction in insulin sensitivity (Ozcan et al. 2004). As a matter of fact, it is interesting to note that UPR can induce a reactivation of HHV8 infection in lymphoma cells by the activation of the XBP-1 factor, which binds to orf50 and activates its promoter (Wilson et al. 2007). UPR is also correlated to the ATF4 cell factor, which is a key protein in the regulation of the response to stress and infection. In a recent study, mice knocked-out for ATF4 were found to be hypo-glycemic, and the lack of the ATF4 factor significantly counteracted the DMT2 induced by a hyper-caloric diet, as well as hyper-lipidemia and hepato-steatosis, suggesting a possible role of ATF4 in glucose metabolism (Yoshizawa et al. 2009). In addition, ATF4 was found to be able to reduce sensitivity to insulin at the tissue level. In this case too, HHV8 infection is able to trigger endothelial reticulum stress in some types of cells, with a consequent over-expression of ATF4, which seems to be important for supporting virus replication (Caselli et al. 2007; Ozcan et al. 2004).

It is known that DMT2 is characterized by the presence of innate immunity acute phase proteins (Pickup et al. 1997; Pickup and Crook 1998),

which can trigger the typical signs of this disease, such as cytokine-induced IR, micro-albuminuria, hyper-cholesterolemia, and hypertension, with the consequent risk of developing cardiovascular complications. A so-called "low-grade-inflammation" is believed to be the cause of the vessel-wall damage that leads to arteriosclerosis (Pickup et al. 1997). This condition is generally mild and has a sub-clinical progression, until the possible development of acute complications, such as heart infarction and strokes. During this phase of the disease, DMT2 subjects can be recognized by screening for acute-phase-proteins, namely C-reactive protein, fibrinogen, and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, leptin and adiponectin). Insulin is known to inhibit acute phase proteins (Pickup and Crook 1998; Thompson et al. 1991): in fact, in experimental diabetic animal models, insulin resistance can enhance the acute-phase response (Campos and Baumann 1992; Pickup et al. 1995; Cabana et al. 1989; Okumura et al. 1985). Moreover, in these animal models, the acute-phase response induces dramatic changes in the lipid metabolism, with increasing triglyceride content and a lowering of HDL cholesterol levels.

Acute-phase proteins are also able to interfere with natural immunity (mainly in NK cells), with a strong impairment of their ability to kill the target cells. The function of NK cells is controlled by the immunoglobulin-like receptors (KIR), which are able to recognize a type of class 1 HLA molecules, thereby inducing a modulation of NK activity (Caselli et al. 2014). DMT2 development is thought to involve environmental, possibly infectious, and genetic factors, and specific killer cell KIR allotypes have been associated with both increased susceptibility to herpes-virus infection and to the risk of developing diabetes. However, no clear gene-disease or virus-disease associations have been established to date. Caselli et al., in collaboration with our Department (Caselli et al. 2014), investigated the possible interplay between HHV8 infection, KIR allotype, and DMT2. Virus prevalence and KIR genotype were analyzed by PCR in a number of patients with DMT2 and several control individuals. Results showed a significant increase in HHV8 prevalence in DMT2 patients versus controls, and a significant increase in KIR2DL2/DS2 homozygosity in diabetes patients infected with HHV8, compared to the uninfected ones. In addition, analysis of the frequency of the KIR2DL2/DS2 receptor and its HLA-C1 ligand showed a significant increased correlation between KIR2DL2/DS2, DMT2, and the HLAC1C1 genotype in the DMT2 patients infected with HHV8, as compared to the uninfected ones. These findings provide additional evidence that HHV8 infection might be a co-factor for DMT2 in genetically susceptible individuals and suggest the possibility that such patients might have an impaired immune-mediated component that contributes to the development of DMT2.

## 5 How Might HHV8 Infection Modify the General Physiological State Leading to the Onset of Diabetes?

Barbara Corkey in her 2011 Banting lecture on diabetes (Corkey 2012), concluded that: "*insulin resistance is an adaptive response, that successfully maintains normal circulating levels of fat and glucose as long as the pancreas β-cell is able to maintain sufficiently elevated insulin levels*". She stressed the important role of diet and physical activity on obesity, but despite evidence to support their utility, these factors have not yet slowed the growth in rates of obesity or diabetes. As a consequence, she proposed the existence of a *Factor X*, which can trigger the metabolic modifications leading to disease and diabetes. She also suggested the following general rules which are at the basis of DMT2: (A) elevated background levels of insulin, superimposed on a susceptible genetic background, or basal hyperinsulinemia are the root cause of insulin resistance, obesity, and diabetes, (B) exposure to free fatty acid affects basal insulin secretion, (C) agents that increase redox or generate ROS result in stimulation of basal insulin secretion.

Based on the previously cited works and Corkey's conclusions, we wonder if there is any

**Scheme 1** Schematic representation of the main steps of the HHV8 replication cycle involved in the cell physiology modifications

| Viral step | Cells and viral factors | Effects on cell physiology |
|---|---|---|
| Human Herpesvirus 8 tropism | Microvascular cells, endothelial cells, B lymphocytes | Cell infection |
| Virus attachment and entry | Cell receptors: Heparan sulfate, Integrins, Efhrin-A2, DC.SIGN (macrophages) | Cell infection, virus replication and ROS production |
| Lytic infection | vIL-6, vGPCR, vIRFs, K1 and K15 (membrane proteins), Insulin Receptor overexpression | Lytic viral replication, membrane permeability alteration, enhanced insulin uptake, immunosuppression |
| Latent infection | LANA, vFLIP, vCyclin, Kaposin (K12) | Latent infection, neutral lipid modification, increased glucose uptake, oncogenesis, angiogenesis |

proof that HHV8 could be considered as a possible *Factor X* in metabolic diseases and diabetes. As a matter of fact, we can state that although there is no definite proof, there are certainly many indicative signs, which can be summarized as follows: (i) HHV8 induces an increase of insulin receptor production and function (and a consequent increased insulin production and consumption) in infected primary human cells (Rose et al. 2007; Ingianni et al. 2013); (ii) HHV8 can infect the pancreas and affect β-cell secretory responses (Sobngwi et al. 2008); (iii) HHV8 infection induces a strong production of ROS in human cells (Bottero et al. 2013; Angius et al. 2015); (iv) HHV8 infection induces an increased synthesis and concentration of lipids in the cell cytoplasm (Bhatt et al. 2012; Delgado et al. 2012; Bottero et al. 2013); (v) HHV8 induces a permanent inflammatory state in the organism, with impairment of B-lymphocyte activity and alteration of NK function (Caselli et al. 2007; Gregory et al. 2012). Scheme 1 summarizes most of these observations and indicates some of the viral factors that can be involved in these physiological modifications.

## 6 Concluding Remarks and Future Prospects

At present, some viruses have been shown to be able to interfere with insulin production and function and can therefore be considered as additional risk factors for the onset of DMT2. While HCV acts directly on liver function and can impair insulin activity and glucose metabolism, HHV8 causes a series of general metabolic modifications, which can lead to altered insulin uptake and neutral lipid accumulation in the cells. In addition, HHV8 induces a general impairment of the immune system, with increased ROS production, a decrease in adaptive immunity, and an up-regulation of both insulin and glucose consumption.

Based on Corkey's considerations (2012) and the cited research findings on HHV8 infection, we suggest that, in particular, HHV8 fits several of the conditions that favor diabetes onset. However, more work is needed in order to transform the reported experimental signs into definite proof that HHV8 could possibly be an example of Corkey's Factor X and that it is involved in DMT2 onset. Further consideration should also be given to the fact that only about 50 % of the DMT2 subjects examined in our studies were permanently infected by HHV8, whilst the other 50 % were free from this virus; this observation implies that other factors (other viruses, either known or unknown?) can be responsible for DMT2 onset in these subjects.

If the hypothesis of a link between DMT2 and HHV8 were to be proved, a broad window would be opened for diabetes prevention and treatment: as a matter of fact, if HHV8 latent infection were a risk factor for diabetes and metabolic syndrome

onset, subjects should be screened for latent HHV8 infection as being at risk of developing diabetes. Screening the population for latent HHV8 would allow subjects at risk of diabetes to be identified before the disease was clinically apparent, so that preventive measures could be taken before diabetes onset. Moreover, specific drugs (several compounds that are able to clear HHV8 from infected cells are already in existence) and a vaccine against HHV8 could be prepared to protect all the subjects at risk (that is to say, 5–10 % of the world population!). Work is in progress in our Department to verify some of these hypotheses.

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# Prevalence of *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Neisseria gonorrhoeae* Based on Data Collected by a Network of Clinical Microbiology Laboratories, in Italy

Maria Cristina Salfa, Barbara Suligoi, and Italian STI Laboratory-based Surveillance Working Group

**Abstract**

Bacterial and protozoal sexually transmitted infections (STIs), such as *Chlamydia trachomatis, Trichomonas vaginalis and Neisseria gonorrhoeae*, may cause acute symptoms, chronic infections and severe long-term complications. The complications of these infections in women include pelvic inflammatory disease, chronic pelvic pain, tubal infertility, ectopic pregnancy, and infertility. Moreover, infection during pregnancy is associated with premature rupture of the membranes, low birth weight and miscarriage.

In Italy, *Chlamydia trachomatis and Trichomonas vaginalis* infections are not subject to mandatory reporting; while *gonorrhoea* is subject to mandatory reporting.

To extend surveillance to STIs that are widespread yet often asymptomatic and to improve the knowledge on the epidemiology of these infections in Italy, in 2009 the "Centro Operativo AIDS of the Istituto Superiore di Sanità", in collaboration with the Association of Italian Clinical Microbiologists (AMCLI, *Associazione Microbiologi Clinici*

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*Italiani*), launched the sentinel STIs surveillance system based on a network of 13 clinical microbiology laboratories.

The main objective of the surveillance was to assess the prevalence and risk factors associated with *Chlamydia trachomatis, Trichomonas vaginalis and Neisseria gonorrhoea* infections among individuals attending microbiology laboratories in Italy.

**Keywords**

Prevalence • Microbiology laboratories • Chlamydia trachomatis • Trichomonas vaginalis • Neisseria gonorrhoeae • Risk factors

## 1 Introduction

Bacterial and protozoal sexually transmitted infections (STIs), such as those by *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Neisseria gonorrhoeae,* may cause acute symptoms, chronic infections and severe long-term complications. The complications of these infections in women include pelvic inflammatory disease, chronic pelvic pain, tubal infertility, ectopic pregnancy, and infertility. Moreover, infection during pregnancy is associated with premature rupture of the membranes, low birth weight and miscarriage. *Chlamydia trachomatis* infection can be transmitted from mother to child during labor, causing perinatal infections. In men, these STIs may cause epididymitis and result in urethral stricture and infertility. Furthermore, individuals with STIs are at increased risk of acquiring or transmitting HIV (Wangnapi et al. 2015; Samarawickrema et al. 2014).

According to the World Health Organization (WHO), over 488 million new cases of three STIs (*Trichomonas vaginalis*; *Neisseria gonorrhoeae*; *Chlamydia trachomatis*) occur worldwide each year in individuals 15–49 years of age (World Health Organization 2012).

European surveillance data show that, in 2012, *Chlamydia trachomatis* infection was the most frequently reported STIs with an overall incidence of 184 cases per 100,000 population; while, *Neisseria gonorrhoeae* was the second most frequently reported STIs with an overall incidence of 15.3 per 100,000 population (European Centre for Disease Prevention and Control (ECDC) 2014).

In Italy, *Chlamydia trachomatis* and *Trichomonas vaginalis* infections are not subject to mandatory reporting. A sentinel surveillance system, in Italy, based on a network of clinical centres specialised in diagnosis and treatment of STIs, collect data on individuals with a symptomatic STI, such as *Chlamydia trachomatis* and *Trichomonas vaginalis* infections. Data provided by this sentinel surveillance system showed that *Chlamydia trachomatis* cervicitis accounted for 8.4 % of all STIs reported among women in 2013, representing the most commonly reported bacterial STIs; whereas *Chlamydia trachomatis* urethritis accounted for 7.2 % of all STIs reported among men in 2013, ranking second among bacterial STIs after primary and secondary syphilis (Salfa et al. 2015). *Trichomonas vaginalis* infection accounted for 0.2 % of all STIs reported among women in 2013; no case was reported among men (Salfa et al. 2015).

In Italy, *gonorrhoea* is subject to mandatory reporting and the number of new cases increased by twofold between 2000 and 2005, and thereafter decreased and stabilized until 2011 (most recent data available) (www.salute.gov.it/malattieInfettive/paginaInternaMenuMalattieInfettive.jsp?id=812&menu=strumentieservizi).

However, mandatory notification of infectious diseases is affected in most countries by underreporting: this shortcoming has been shown for syphilis and gonorrhoea in Italy in a previous study (Greco et al. 1990).

To extend surveillance to STIs that are widespread yet often asymptomatic and to improve the knowledge on the epidemiology of these infections in Italy, in 2009 the "Centro Operativo

AIDS of the Istituto Superiore di Sanità", in collaboration with the Association of Italian Clinical Microbiologists (AMCLI, *Associazione Microbiologi Clinici Italiani*), launched the sentinel STIs surveillance system based on a network of 13 clinical microbiology laboratories.

The main objective of the surveillance was to assess the prevalence and risk factors associated with *Chlamydia trachomatis* (Ct), *Trichomonas vaginalis* (Tv) and *Neisseria gonorrhoea* (Ng) infections among individuals attending microbiology laboratories in Italy.

## 2 Methods

### 2.1 Source of Data

Cases reported by these laboratories involved individuals who were asymptomatic or symptomatic (i.e. absence or presence of genital symptoms) (Salfa et al. 2015). The biological samples were obtained from individuals who underwent testing for one or more of the three infections. For each individual, up to all three infections could be reported (i.e. mixed infections). Laboratories received a request to perform a test for one or more of the three infections from healthcare providers (i.e. general practitioners, dermatologists, gynaecologists, urologists, family planning centres, infertility centres, STIs clinics, medical doctors working in hospital, and others). In addition, there were individuals who accessed the laboratory directly.

Healthcare providers collected socio-demographic (i.e. gender, age, nationality or continent of origin), behavioural (i.e. number of sexual partners in the previous 6 months) and clinical (i.e. pregnancy at the time of sample collection, presence of genitourinary symptoms at the time of sample collection) data for every individual. Laboratories collected the same data for individuals who accessed the laboratory directly.

This information was collected on all individuals, either positive or negative for any infection.

Data were sent by participating laboratories to the Centro Operativo AIDS of the Istituto Superiore di Sanità through a web-based platform. All individuals provided written informed consent for participation.

### 2.2 Criteria Used to Select the 13 Participating Laboratories

The criteria for microbiology laboratory selection were as follows:

- geographic representativeness (6 laboratories located in the north, 3 in the center, and 4 in the south of Italy);
- more than 500 tests per year performed for the three STIs;
- capability to collect individual socio-demographic, behavioural and clinical information;
- use of high-standard laboratory methods (see "Laboratory methods").

### 2.3 Laboratory Methods

Participating laboratories complied with the following minimum criteria for the diagnosis of pathogens:

(a) use of nucleic acid amplification tests (NAAT) for Ct; and
(b) use of microscopic identification and/or cultural isolation and/or immunochromatography and/or NAAT for Tv; and
(c) use of cultural isolation and/or NAAT for Ng.

### 2.4 Sample Collection

For Ct infection, laboratories performed NAAT on samples taken from the genital or anal area. The test could be performed on the first voided urine or the peritoneal fluid.

For Ng infection, the laboratories performed cultural isolation and/or NAAT on samples taken from the genital or anal area and/or the seminal fluid in males. The NAAT could be performed on the first voided urine.

For Tv infection, the laboratories performed microscopic identification on samples taken from the urethra in males and the vagina in females (this method could only be used if the microscope analysis was performed immediately following sample collection); alternatively, laboratories could perform cultural isolation and/or immunochromatography on samples taken from the genital or anal area, or the seminal fluid in males. NAAT could be used on samples taken from genital, anal area, seminal fluid, or first voided urine.

### 2.5 Statistical Analysis

Prevalence was calculated as the ratio between the number of individuals affected by Ct or Tv or Ng infection and the overall number of individuals tested for Ct or Tv or Ng. The Chi-square test was used to investigate associations between Ct, Tv and Ng infections and risk factors. To calculate the crude odds ratio (cOR) for each factor, a univariate logistic regression model was used. Multivariate analyses (adjusted OR, aOR) were conducted using a binary logistic regression model; variables included in the model were those that were statistically significant at the univariate analysis (p-value $<0.05$ and p-value $<0.001$). Records with missing data in any of the variables were excluded from the regression modelling.

The analysis was performed using IBM SPSS Statistics 22.

## 3 Results

From 1 April 2009 to 31 December 2013, the laboratories collected 93,403 biological samples, which were tested for at least one of the three infections. Of the individuals who provided samples, 87.7 % (No. 81,886) were women, and 12.3 % (No. 11,517) were men.

The median age was 35 years [inter-quartile range (IQR) = 29–40 years]; it was 34 years for women (IQR = 29–40 years) and 37 years for men (IQR = 30–44 years).

Of the individuals who provided samples, 15.0 % (No. 13,906) were migrants.

Most of the samples were request by general practitioners (44.3 %), gynaecologists (25.3 %), medical doctors working in hospital (8.3 %), and individuals who accessed the laboratory directly (6.5 %).

Nearly one quarter of women (28.1 %) were pregnant.

About half of the individuals (49.4 %) did not present genitourinary symptoms at the time of sample collection.

Regarding behaviour, 5.3 % of all individuals reported that they had had more than one sexual partner in the previous 6 months, whereas 94.7 % had had one or no sexual partner.

Of the 93,403 total tests performed, 83,000 (88.9 %) were for Tv, 70,680 (75.7 %) for Ct and 50,822 (54.4 %) for Ng (Table 1).

### 3.1 *Chlamydia trachomatis* Infection

The number of individuals tested for Ct increased between 2009 and 2010, and then remained relatively stable (Fig. 1). The prevalence of Ct infection was stable between 2009 and 2012, and showed a slight increase in 2013 (Fig. 1).

Among women, Ct tests were most frequently requested by general practitioners (37.0 %), gynaecologists (31.2 %), and medical doctors working in hospital (9.8 %) whereas among men, by general practitioners (37.0 %), fertility centres (15.6 %) and STIs clinics (11.3 %).

The overall prevalence of Ct infection was 3.2 % (2,281 cases) (Table 1).

#### 3.1.1 Prevalence of Ct Infection

The prevalence of Ct infection was significantly higher (p-value $<0.001$) among men (Table 1), and among young persons (15–19 years of age), compared to older persons (8.2 % vs. 3.1 %; p-value $<0.001$) (data not shown). In particular, the prevalence of Ct infection significantly decreased by increasing age group ($X^2$ linear trend 582,31869;

**Table 1** Prevalence and risk factors for *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Neisseria gonorrhoeae* infections

| | *Chlamydia trachomatis* | | | *Trichomonas vaginalis* | | | *Neisseria gonorrhoeae* | | |
|---|---|---|---|---|---|---|---|---|---|
| Characteristic | N. | Prevalence (%) | aOR (95 % CI) | N. | Prevalence (%) | aOR (95 % CI) | N. | Prevalence (%) | aOR (95 % CI) |
| **Total tested** | 70,680 | **3.2** | – | 83,000 | **0.7** | | 50,822 | **0.5** | |
| **Gender** | | | | | | | | | |
| Men | 9,674 | **8.4** | 2.8 (2.6–3.2) | 7,427 | **0.1** | 7.8 (3.5–17.6) | 10,243 | **2.1** | 11.3 (7.6–16.9) |
| Women | 61,006 | **2.4** | 1 | 75,573 | **0.8** | 1 | 40,579 | **0.1** | 1 |
| na | 0 | – | | 0 | – | | 0 | – | |
| **Age (in years)** | | | | | | | | | |
| 15–19 | 1,949 | **8.2** | * | 2,040 | **0.5** | ** | 1,399 | **0.5** | ns |
| 20–24 | 7,186 | **8.1** | | 7,731 | **0.6** | | 5,059 | **0.7** | |
| 25–44 | 53,163 | **2.6** | | 61,963 | **0.6** | | 37,622 | **0.4** | |
| ≥45 | 8,343 | **2.0** | | 11,224 | **1.4** | | 6,727 | **0.7** | |
| na | 39 | **0.0** | | 42 | **0.0** | | 15 | **0.0** | |
| **Nationality** | | | | | | | | | |
| Italians | 58,840 | **3.2** | ns | 69,785 | **0.6** | 1 | 42,808 | **0.5** | ns |
| Migrants | 11,327 | **3.4** | | 12,687 | **1.5** | 2.7 (2.2–3.3) | 7,806 | **0.4** | |
| na | 513 | **4.5** | | 528 | **0.6** | | 208 | **1.4** | |
| **Current pregnancy***° | | | | | | | | | |
| Yes | 15,478 | **1.2** | 1 | 21,935 | **0.5** | ns | 10,783 | **0.1** | ns |
| No | 44,428 | **2.8** | 1.7 (1.4–2.0) | 52,404 | **0.9** | | 29,414 | **0.1** | |
| na | 1,100 | **5.6** | | 1,234 | **1.9** | | 382 | **0.3** | |
| **Genitourinary symptoms at the time of sample collection** | | | | | | | | | |
| Yes | 3,695 | **4.5** | 1.8 (1.6–2.0) | 40,730 | **1.1** | 2.7 (2.2–3.3) | 24,366 | **0.9** | 4.6 (3.0–7.0) |
| No | 35,993 | **2.0** | 1 | 39,603 | **0.4** | 1 | 25,253 | **0.1** | 1 |
| na | 1,982 | **3.8** | | 2,667 | **1.0** | | 1,203 | **0.7** | |
| **Number of sexual partners in previous 6 months** | | | | | | | | | |
| 0–1 | 60,082 | **2.2** | 1 | 69,277 | **0.6** | 1 | 41,027 | **0.2** | 1 |
| ≥2 | 3,826 | **13.4** | 4.4 (4.0–5.0) | 2,978 | **1.0** | 1.5 (2.2–3.3) | 2,936 | **2.8** | 5.3 (3.9–7.3) |
| na | 6,772 | **6.4** | | 10,745 | **1.4** | | 6,859 | **1.2** | |

*na* not available, *ns* not significant

*°Percentages based on total number of women

*See Sect. 3.1.2

**See Sect. 3.2.2

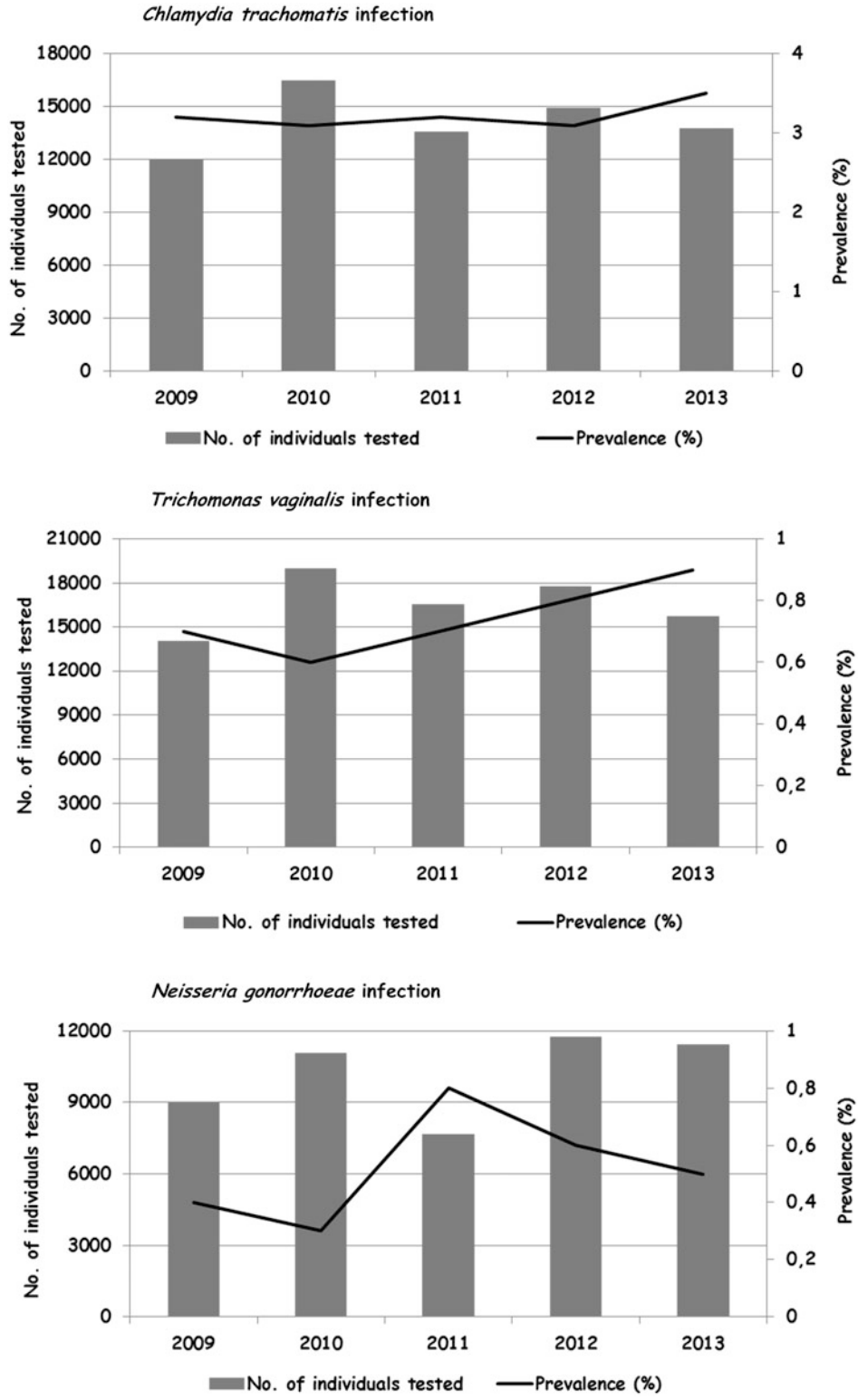


**Fig. 1** Prevalence of *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Neisseria gonorrhoeae* infections

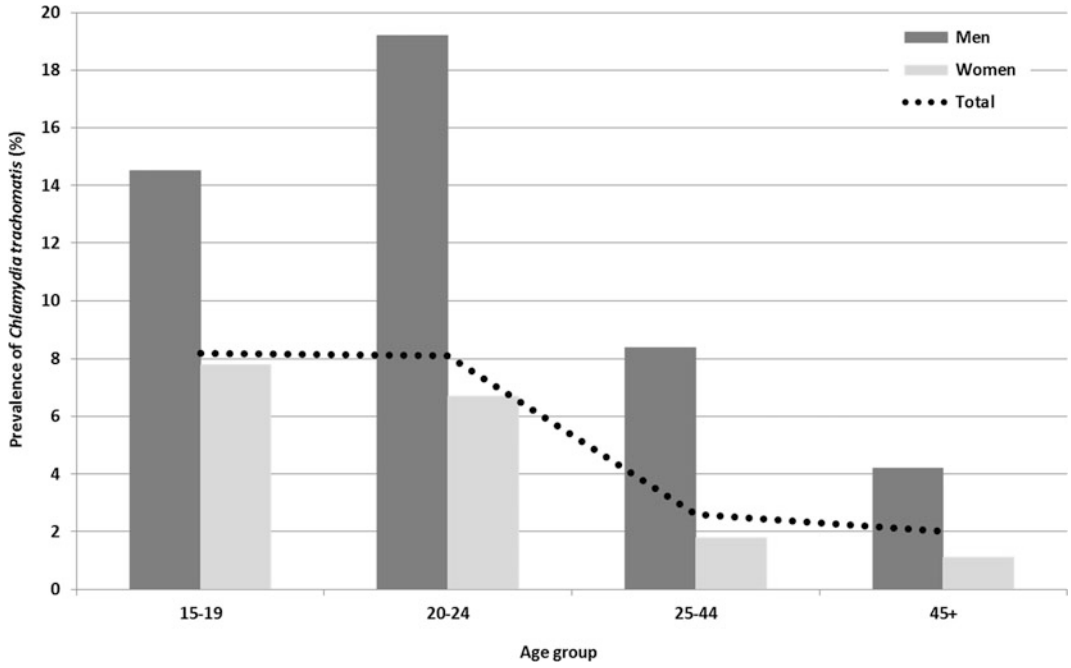


**Fig. 2** Prevalence of *Chlamydia trachomatis* infection, by age group and by gender

p-value <0.001), from 8.2 % among 15–19 year-old individuals, to 2.0 % among individuals older than 44 years (Fig. 2, Table 1). The prevalence was higher among males in all age groups (Fig. 2).

Moreover, the prevalence of Ct infection was significantly higher (p-value <0.001) among not pregnant women, individuals with genito-urinary symptoms at the time of sample collection, and among persons who reported more than one sexual partner in the previous 6 months (Table 1).

One third (32.7 %) of the persons with Ct infection did not show genitourinary symptoms at the time of sample collection. The percentage of asymptomatic individuals was significantly higher among women (36.4 % vs. 27.7 % among men; p-value <0.001) and pregnant women (57.1 % vs. 33.4 % among non-pregnant women; p-value <0.001).

#### 3.1.2 Risk Factors Significantly Associated with Ct Infection

In the multivariate analysis (Table 1), factors independently associated with Ct infection were: male gender; young age (15–19 years vs. ≥20 years) [aOR 2.8 (95 % CI 2.3–3.4); data not shown]; not being pregnant; presence of genitourinary symptoms at the time of sample collection; and having had more than one sexual partner in the previous 6 months.

### 3.2 *Trichomonas vaginalis* Infection

The number of individuals tested for Tv increased between 2009 and 2010, and then remained relatively stable (Fig. 1). The prevalence of Tv infection increased between 2010 and 2013 (Fig. 1). The overall prevalence of Tv infection was 0.7 % (606 cases) (Table 1).

#### 3.2.1 Prevalence of Tv Infection

The prevalence of Tv infection was significantly higher (p-value <0.001) among women; and among old persons (≥45 years of age), compared to younger persons (1.4 % vs. 0.6 %; p-value <0.001) (data not shown). Moreover, the

prevalence of Tv infection was significantly higher (p-value <0.001) among migrants, not pregnant women, individuals with genitourinary symptoms at the time of sample collection and among persons with more than one sexual partner in the previous 6 months (p-value <0.05) (Table 1).

#### 3.2.2 Risk Factors Significantly Associated with Tv Infection

In the multivariate analysis (Table 1), the factors independently associated with Tv infection were: female gender, older age (≥45 years vs. 15–44-years) [aOR 2.1 (95 % CI 1.6–2.6); data not shown]; being migrant; presence of genitourinary symptoms at the time of sample collection; and having had more than one sexual partner in the previous 6 months.

### 3.3 *Neisseria gonorrhoeae* Infection

During the study, the number of individuals tested for Ng decreased between 2010 and 2011, then increased until 2013 (Fig. 1). The prevalence of Ng infection increased between 2010 and 2011, then decreased until 2013 (Fig. 1). The overall prevalence of Ng infection was 0.5 % (258 cases) (Table 1).

#### 3.3.1 Prevalence of Ng Infection

The prevalence of Ng infection was significantly higher (p-value <0.001) among men; individuals with genitourinary symptoms at the time of sample collection; and among persons with more than one sexual partner in the previous 6 months (Table 1).

#### 3.3.2 Risk Factors Significantly Associated with Ng Infection

In the multivariate analysis (Table 1), the factors independently associated with Ng infection were: male gender; presence of genitourinary symptoms at the time of sample collection; and having had more than one sexual partner in the previous 6 months.

## 4 Discussion

This is the first Italian study providing data on three important STIs (Ct, Tv and Ng infections) involving 13 large microbiology laboratories, combining detailed individual data collection with diagnosis based on molecular amplification methods.

This study provided the total number of individuals tested disregarding positivity or negativity to laboratory tests; which is important for the calculation of prevalence rates.

The prevalence of Ct infection among men was similar to that reported in other high-income countries (such as Canada and Australia) among men of similar median age (Redmond et al. 2015).

The prevalence of Ct infection among women was similar to that reported in previous Italian studies (Grio et al. 2004; Salfa et al. 2011a), and in studies conducted recently in the European Union/European Economic Area Member States (such as the United Kingdom and the Netherlands) and in other high income countries (such as USA) (Redmond et al. 2015).

The association of Ct infection with young age (≤25 years), which has also been reported in other countries (such as the United Kingdom, Slovenia, France, Germany, Denmark) (Redmond et al. 2015; Fenton et al. 2001; Wilson et al. 2002; Kucinskiene et al. 2006; Goulet et al. 2010; Desai et al. 2011; Adams et al. 2004), is probably due to the fact that genital tissues are still immature and therefore more susceptible to pathogens (Fenton et al. 2001; Stevens-Simon and Sheeder 2005).

The negative association with pregnancy has been reported also in some not recent studies conducted in Belgium and Australia, whereas one study conducted in USA reported an increased association with pregnancy probably due to some level of immune suppression that is known to occur in pregnant women (Navarro et al. 2002).

The association between Ct infection and a high number of sexual partners in the previous 6 months suggests that sexual exposure with multiple partners constitutes a risk factor, as confirmed by a number of studies in Italy and other European countries (Grio et al. 2004; Fenton et al. 2001; Goulet et al. 2010; Salfa et al. 2011b).

The prevalence of Tv infection was higher among women, possibly due to a higher susceptibility among females associated with a longer persistence of infection, the effect of female hormones and menstrual bleeding (Poole and McClelland 2013).

The association of Tv infection with older age (≥45 years), which has also been reported in other countries (Sutton et al. 2007; Verteramo et al. 2008; Geelen et al. 2013; Mitchell et al. 2014), may be due to a long duration of infectiousness (Verteramo et al. 2008). Our findings show that the prevalence of Tv infection was higher among migrants compared to Italians as reported from Greece and England (Mitchell et al. 2014; Piperaki et al. 2010). Moreover, differences in religious, social and cultural factors may influence personal hygiene standards and sexual behaviour, and are considered as major risk factors for Tv infection (Piperaki et al. 2010).

The positive association of Tv infection with genitourinary symptoms at the time of sample collection has been reported in some studies conducted in Australia, Brazil and Croatia among women and men, and is probably due to the association of Tv infection with other STIs. Indeed, Tv infection can be used as a marker for STIs sexual risk behaviour (Piperaki et al. 2010; Grama et al. 2013; Uddin et al. 2011; Sviben et al. 2015).

The association of Tv infection with high number of sexual partners, as confirmed by other studies conducted in Italy and USA (Sutton et al. 2007; Verteramo et al. 2008), is probably related to the fact that the sexual promiscuity together with low immunity and laxity of sexual health may predispose to STI.

The finding that the prevalence of Ng infection was higher among men compared to women is consistent with a study conducted in the United Kingdom among young persons (15–24 years) (Rao et al. 2008), suggesting that they were more likely to go to the physician once they knew to be affected by a STI. In fact, in the study population, a high percentage of men with Ng infection were symptomatic.

The association of Ng infection with sexual promiscuity, was confirmed by other studies conducted in United Kingdom, Wales, Ireland, Sweden and Denmark (Fenton and Lowndes 2004). In the population described herein, the prevalence of Ng infection was also higher among symptomatic individuals, as reported by another study conducted in Brazil (Barbosa et al. 2010), confirming that Ng infection is largely symptomatic.

Some limits of the sentinel surveillance system should be addressed. The system can be used to evaluate trends in the occurrence of a given event in a given area, unlike systems based on mandatory reporting, which aim to collect information on all cases. Although the sentinel surveillance system may only capture a low percentage of the infections occurring in a specific area or country, the selection of the microbiology laboratories in this study ensured that reliable information have been provided on a stable percentage of cases over time.

The above described sentinel surveillance system shows some specific advantages: low cost (laboratory participation in the network is voluntary with no economic compensation); no selection of enrolled individuals (individuals are not pre-selected by the presence of specific symptoms as they can be asymptomatic or symptomatic, attending for a routine check-up, compared to STIs clinic specialists that visit mainly symptomatic patients); detailed information (microbiology laboratories report data on the number and characteristics of participants, providing also a denominator necessary to estimate the burden of Ct or Tv or Ng infection, in terms of frequency and prevalence); high diagnostic standards; real-time transmission of data; centralized data management and analysis.

The results of the study stress the need to promote information campaigns about the clinical presentation and the complications of Ct, Tv and Ng infections; to promote safe sex (including the correct and consistent use of condoms or reducing the number of sexual partners); to implement early diagnosis, through screening, which involves testing individuals who are not directly seeking any health care (i.e. Ct screening among young individuals); and enhance treatment prompt, that the chain of transmission and

prevents the development of complications and long-term sequelae; to improve partner notification and contact tracing; to promote treatment of sexual partners; and to support a behavioural surveillance for monitoring trends in risk behaviour over time.

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# β-Defensins: Work in Progress

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**Abstract**

Defensins are a group of antimicrobial peptides (AMPs) found in different living organisms, and are involved in the first line of defense in the innate immune response against pathogens. The increase in the resistance of bacteria to conventional antibiotics and the need for new antibiotics has stimulated interest in the use of AMPs as new therapeutic agents. The inducible nature of human defensin genes suggests that it is possible to increase the endogenous production by utilizing small molecules of various origins to enhance, even selectively, the expression of these peptides. In the light of their role in immunomodulation, angiogenesis, wound healing, inflammation and cancer, as well as their antimicrobial activity, it is possible induce their expression or create analogs with increased specific activity or various degrees of selectivity, or obtain human defensins with genetic engineering to optimize the potency and safety in order to reduce cytotoxicity and potential proinflammatory activity and susceptibility to protease and salt. Restoring the balance between immunostimulating and immunosuppressive molecules may be an important strategy to correct expression defects in specific diseases.

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## 1 Introduction

In the course of evolution, host defense mechanisms have developed that include both the constitutively expressed innate natural defenses, and adaptive, specifically induced defensins. Innate immunity is involved in the early control of infections. The several defenses of the human body include physical systems such as structural integrity, flow of saliva, peristalsis, cell-shedding, flushing of the urinary tract, keratinized epithelia (skin, mouth and vagina) and chemical defenses such as low pH in the stomach, vagina and skin, as well as the salt and lipids produced by the skin and a wide variety of antimicrobial molecules. The main components of innate immunity are the epithelia, the products of epithelial cells, phagocytes, normal microbiota, the mediators of the acute phase as well as endothelial cells, mast cells, basophils, fibroblasts, platelets and cell populations in the mucosa epithelia, such as M cells, Paneth cells and intraepithelial lymphocytes.

The surfaces exposed such as the skin and mucosa, the first line of defense in which the epithelial cells play a key role, are covered with a protective coating that is an important microenvironment interface whose integrity is essential to prevent the penetration of microorganisms. The chemical defenses in the skin and mucous membranes are entrusted to a number of antibacterial peptides and proteins that make up a list destined to grow more and more (Breitkreutz et al. 2009; Eckert et al. 2006; Proksch et al. 2008; Baroni et al. 2012; Svanborg et al. 1999; Travis et al. 2001). Some of these molecules have received considerable attention in recent years.

In recent decades, a large number of antimicrobial peptides (AMPs) able to rapidly kill bacteria, fungi and viruses have been isolated from a wide range of organisms, plants, insects, amphibians and mammals. Although varying considerably in the number of amino acid residues (10–50 amino acids) and in the structure, they show common features. AMPs are structurally heterogeneous and are mainly cationic, of an amphipathic nature, synthesized in the form of propeptides and released as a mature form after the action of specific proteases. The fact that these peptides are also present in the lower phyla suggests that they are the product of a convergent evolution. Antimicrobial peptides and cytokines are a "functional module" of the innate immune system (Radek and Gallo 2007) and also communicate with cells of the adaptive immune system. In mammals the most studied AMPs are human defensins and cathelicidins.

This review focuses specifically on the role of human defensins in human mucosal immunity and cites both old and new data relating to their biological activities and their role in health and disease.

The defensin family comprises three subfamilies (α, β and θ defensins) and is one of the groups of antimicrobial peptides involved in the innate immune response. Human α-defensins are stored in granules of PMN (HNP 1–4) or epithelial granulocytes of the gut (HD-5, HD-6). They differ from β-defensins for the different position of the disulfide bond and different spectrum of antimicrobial activity. While α-defensins have a very broad spectrum of microbicidal activity and act on Gram-positive and Gram-negative bacteria, fungi and the envelope of some viruses, β-defensins act mainly against Gram-negative bacteria and fungi. The similarity of the amino acid sequence, their tertiary structure and location of all α-defensin and most β-defensin genes in the p22–23 regions on

the same chromosome (chromosome 8 in humans) indicate a common origin of both defensin subfamilies. Both classes of defensins exert their action by interacting with the outer surface of microorganisms; in the case of Gram-negative bacteria they bind to the portion of the polyanionic lipopolysaccharide, while in the case of Gram-positive bacteria they interact with teichoic acid or with anionic groups positioned externally to the layer of peptidoglycan. The interaction of defensins with the different molecules of the bacterial surface leads to a permeability of the surface, either through a detergent effect that destabilizes the bonds, resulting in leakage of the cytoplasmic components, or through the formation of channels due to the aggregation of monomers of the peptide, which form pores once the outer surface is "carpeted".

## 2 Biology of Human Defensins

The family of β defensins is composed of cationic, amphiphilic peptides (~4 kDa), with a β-sheet structure. The β defensins are produced by epithelial cells, constitutively (human β defensin HBD-1) or induced by microorganisms or cytokines. HBD-1 is considered the most important antimicrobial peptide in epithelial defense against infection, is the only β-defensin gene with a constitutive expression in skin keratinocytes and urinary and respiratory tract epithelial cells, and is, in addition, capable of upregulation through microbial or inflammatory stimuli. The highest HBD-1 concentrations have been found in the kidney and female reproductive tract, especially in pregnant women. Other expression sites include epithelial cells of the testis, gingival tissue, small intestine, cornea, mammary gland, and astrocytes (Pazgier et al. 2006). The HBD-1 knockout mouse model exhibits significantly higher counts of *Staphylococcus* sp. in the bladder than in wild-type counterparts and less efficient lung clearance, especially at higher *Haemophilus influenzae* doses (Pazgier et al. 2006). Both HBD-1 and HBD-2 selectively chemoattract human immature dendritic cells (iDCs) and memory T-cells *in vitro*. Their chemotactic activities involve the chemokine receptor CCR6, which mediates innate signaling from innate through adaptive immunity. In addition to its chemoattraction properties, HBD-1 may also be involved in DC maturation and/or activation via CCR6 and pattern recognition receptors such as toll-like receptors (TLRs), probably having a marked effect on skin inflammation and/or skin responsiveness in some allergic reactions (Prado-Montes de Oca et al. 2007). HBD-2 is an inducible antimicrobial peptide, identified in psoriatic lesions as the most abundant AMP. In the skin it is localized in the uppermost layers of the epidermidis and/or stratum corneum in lamellar body granules (Harder et al. 1997). HBD-2 is present in various other epithelia, such as the epithelia of the oral cavity (Dale and Krisanaprakornkit 2001), gingival epithelial cells, the paranasal sinuses (Carothers et al. 2001), the corneal epithelia (McDermott et al. 2003) and in intestinal, respiratory and urogenital epithelial cells. HBD-2 is stimulated by proinflammatory cytokines through MAP-kinase and NF-kB, is upregulated by infections (Radek and Gallo 2007) or wounds (Butmarc et al. 2004) and is sensitive to the concentration of NaCl (Bals et al. 1998). In cultured colon epithelial cells HBD-2 is induced by proinflammatory cytokines, such as IL-1α, or by invasive microorganisms. HBD-2 protects the renal tubular epithelial cells against pathogens in the urinary tract. IL-1α and β, *Pseudomonas* and LPS induce HBD-2 in keratinocytes and respiratory epithelia (Harder et al. 2000; Liu et al. 2002, 2003). Various cytokines modulate HBD-2 expression in alveolar macrophages and monocytes. HBD-2 is chemoattractant for neutrophils (Niyonsaba et al. 2004), for I DCs and memory T-cells; these activities are mediated by interaction with CCR6 (Yang et al. 1999). HBD-2 is a chemotaxin for mast cells and induces histamine release and prostaglandin synthesis, which both play a role in allergic responses (Niyonsaba et al. 2001). TLR-2-mediated recognition of *S. epidermidis* increases the expression of β defensins 2 and 3, activating host immunity to *S. aureus* infection

(Lai et al. 2010; Wanke et al. 2011). Lipoteichoic acid from *S. epidermidis* increases skin mast cell antimicrobial activity against vaccinia virus through the release of cathelicidin antimicrobial peptide (CAMP). Thus, commensal microbes show an important role in amplifying the host immune defenses against pathogens (Sanford and Gallo 2013). HBD-3 is identified in psoriatic scales (Harder et al. 2001) and is expressed in the skin, placenta and oral tissue (Harder et al. 2001; Dunsche et al. 2002). It is for 43 % identical to HBD-2 and shares the β-defensin six cysteine motif (Harder et al. 2001; Corrales-Garcia et al. 2011). HBD-3 shows antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi (Harder et al. 2001) and is active against multiresistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* (Harder et al. 2001). The activity increases in combination with other antimicrobial agents such as amoxycillin and chlorhexidine (Maisetta et al. 2003). HBD-3 is detected in the endometrial epithelium. Proinflammatory cytokines and growth factors (EGF-1 and TGF-α) increase the expression of HBD-3 in keratinocytes. IFN-γ is the most powerful HBD-3-inducing cytokine (Harder et al. 2004). Epidermal growth factor GF induces the expression of HBD-3 in keratinocytes during proliferation in skin wounds (Sørensen et al. 2006). HBD-4 contains an amino acid sequence identical to HBD-1, HBD-2 and HBD-3 for 20–25 %. Genomic analysis revealed AP-1 and GAPA-1 as binding sites, but not NF-kB or STAT (Corrales-Garcia et al. 2011). Also HBD-4 is expressed in the human endometrium with a peak in the proliferative phase of the menstrual cycle and is highly expressed in the testes and gastric antrum. HBD-4 is inducible by microorganisms in epithelial cells and kills *E. coli*, *S. faecalis* and *C. albicans*. The antibacterial activity vs. *E. coli* is 100 times compared to other HBDs.

## 3 Defensins and Skin Disease

Human β-defensins such as HBD-2 and HBD-3, like other antimicrobial peptides, play an important role in skin diseases. The most common skin diseases characterized by chronic inflammation are atopic dermatitis (AD) and psoriasis. Genetic, immunological and environmental factors play an important role, as well as skin barrier abnormalities. Thirty percent of patients with AD suffer from recurrent skin infections whereas only 6.7 % of psoriatic patients have these complications (Christophers and Henseler 1987). The prevalence of infections in AD is associated with a reduced expression of HBD-2 and HBD-3 (Ong et al. 2002; Howell et al. 2005, 2006) compared to an over-expression of antimicrobial peptides, such as HBD-2, in psoriasis. AD is dominated by Th2 cytokines (IL-4, IL-5, IL-13), which reduce the expression of AMPs, especially that of the β-defensins.

Psoriasis is dominated by Th 1 cytokines (IFN-γ, TNF-α), which stimulate proliferation and differentiation of keratinocytes and induce AMP expression (Grossman et al. 1989; Nomura et al. 2003). There is evidence for an association between the higher genomic copy number of HBD-2 genes and the risk of psoriasis (Hollox et al. 2008).

In AD, there is increased susceptibility to infections, particularly bacterial but also viral and mycotic. The decrease in ceramides, filaggrin mutations and keratinization abnormalities promote the penetration of microorganisms. Recently Nakajima et al. (2013) studied the effects of ceramide deficiency in the epidermidis of mice, which impaired the water-holding capacity and barrier function. The mice developed psoriasis-like lesions and showed an upregulation of psoriasis-associated genes that express defensins, psoriasins, IL-17 and IL-22, which favored hyperkeratosis and inflammation.

It is reported that HBD-2, and in some cases also HBD-3, plays an important role in innate immunity in mycotic diseases. However, little is known about how fungi overcome the natural barrier of the skin.

The lipophilic yeast *Malassezia furfur* is a saprophyte found in normal human cutaneous flora. *Malassezia furfur* is also associated with several diseases such as pityriasis versicolor, folliculitis, seborrheic dermatitis and some

forms of atopic dermatitis, psoriasis and confluent and reticulate papillomatosis (Ashbee and Evans 2002).

We have shown that *M. furfur* can surpass the skin barrier by penetrating HaCat keratinocytes via an endocytic process, thus inhibiting transglutaminase. The inhibition of phagolysosome formation and of proinflammatory cytokines might allow the yeast to survive in the host cell without causing any inflammatory response (Baroni et al. 2001).

The invasive ability of *M. furfur* in keratinocytes after 48 h is accompanied by an upregulation of HBD-2 via protein kinase C (PKC), involvement of AP-1 and no modification of HBD-1 expression (Donnarumma et al. 2004). In addition, an over-expression of IL-10 and TGFβ-1 is induced in *M. furfur*-treated HaCat cells, suggesting that these cytokines, which interfere with the development of protective cell immunity, enable *M. furfur* to persist and multiply in keratinocytes. The over-expression of TGFβ-1 also induces the keratinocytes to express integrins αvβ5 and αvβ1 (Baroni et al. 2003) and HSP-70, molecules involved in cell migration and hyperproliferation with loss of polarization. In hyperproliferative skin diseases, such as skin cancer (De Luca et al. 1994; Rolli et al. 2003; Baroni et al. 2004) or psoriasis vulgaris, the polarized expression is lost. This loss could help explain the role played by *M. furfur* in psoriasis. TLR-2 mediates the recognition of *M. furfur* and induces an upregulation of HBD-2, favoring skin defense (Baroni et al. 2006). *M. furfur* also upregulates the expression of TLR-1, as well as HBD-3 and IL-8. The same results were obtained *in vivo* using psoriatic skin biopsies (Baroni et al. 2004). MyD88, a protein required for intracellular signaling of some TLRs is also increased in *M. furfur*-treated keratinocytes. In conclusion, protection against *M .furfur* depends on the HBD-2 production, which, in addition to a direct antimicrobial effect for its chemotactic activities, plays an important role in adaptive immunity and wound healing.

*M. globosa* and *M. restricta* are most commonly isolated from the scalp of individuals with dandruff and/or scalp seborrheic dermatitis (Clavaud et al. 2013). Donnarumma et al. (2014a), studying the interaction of human keratinocytes with *M. globosa* and *restricta* and the importance of the different growth phase, showed different modulation of the inflammatory and immunomodulatory response compared to *M. furfur*. *M. globosa* and *restricta* induced an early expression of inflammatory markers and a shift in cytokine induction from pro-inflammatory towards immunoregulatory in the late phase of infection. In addition, *M. restricta* showed the expression of TLR-2-independent IL-8 and a lesser increase in immunomodulatory cytokines IL-10 and TGF-β, suggesting a limited control of inflammation that could partly explain its greater virulence.

*Malassezia pachydermatis,* a normal inhabitant of canine and feline skin, may be associated with feline otitis externa. Few reports have demonstrated the isolation of this yeast from human patients (Prohic and Kasumagic-Halilovic 2009). Watenabe et al. (2001) studied the cytokine production in human keratinocytes cultured in the presence of *M. pachydermatis* and showed higher levels of cytokines compared to other *Malassezia* species.

Recently Buommino et al. (2013) studied *in-vitro* interaction between human keratinocytes and a clinical strain of live *M. pachydermatis* isolated from an otitic cat. They showed the ability *of M. pachydermatis* to invade HaCat cells and to activate TLR-2 expression. This activation upregulates the HBD-2 gene expression in the inflammatory response, with a strong induction of IL-1β, TNFα, IL-6 and IL-8, demonstrating the important role played by HBD-2 in the immune innate response of keratinocytes, as already reported for *M. furfur* (Donnarumma et al. 2004). In addition, the inflammatory response induced by *M. pachydermatis* is a defense mechanism of the human skin against this yeast. These results may indicate a role of *M. pachydermatis* as a human opportunistic pathogen.

## 4 Defensins and Gastrointestinal Disease

The intestinal colonizing microbiota is essential for homeostasis. Host factors are important to regulate the composition of the ecosystem. Innate immunity, in particular via AMPs, plays an essential role in maintaining the balance between protection from pathogens and tolerance to the normal microbiota, as their deregulation has deleterious effects and shifts this balance towards inflammation, resulting in inflammatory bowel disease (IBD). Infections or injuries cause the over-expression of AMPs, which disrupts the microbial membranes and stimulates the recruitment of immune cells to allow a return to homeostasis. Colon epithelial cells constitutively express HBD-1. The expression of HBD-2, 3 and 4 is induced by various inflammatory and bacterial stimuli. Colon plasma cells also express HBD-2, 3 and 4. IBD is an idiopathic, chronic and relapsing inflammatory condition of the gastrointestinal tract. Crohn's disease (CD) and ulcerative colitis are subgroups of IBD. CD, which involves genetic susceptibility and environmental influences, is a chronic inflammatory disease involving the mucosa of the distal small bowel and portions of the ascending colon. Inflammation occurs as an abnormal response to commensal flora (Shanahan 2002). HBD-2 expression is markedly depressed in the enterocytes of the ileum and colon in subjects with CD, compared to patients with ulcerative colitis (UC) and non-specific colitis (Wehkamp et al. 2002). Changes in the colonizing microbiota may mediate this pathogenic mechanism. Low levels of defensins in Paneth cells may predispose premature neonates to necrotizing enterocolitis (NEC), which shows the role of defensins in the neonatal and fetal period before the presence of enteric bacteria (Bevins 2006). Inflammatory conditions such as *Helicobacter pylori*-associated gastritis regulate the expression of two major antimicrobial peptides β defensins 2 and 3. *H. pylori* and IL-1β have been shown to be effective inducers of HBD-2 (Bajaj-Elliott et al. 2002), and *H. pylori* is susceptible to human β-defensins 2 and 3 (Hamanaka et al. 2001; Uehara et al. 2003; George et al. 2003). HBD-2 expression is induced in a Cag PAI-dependent manner via the Nod-1-dependent NF-kB pathway (Hornsby et al. 2008; Grubman et al. 2010), while HBD-3 is induced via EGFR-dependent activation of MAPKs and STATs (Bauer et al. 2012). Cover and Blaser (2009) showed that *H. pylori* CagA reduces the expression of HBD-3 via SHP-2-dependent EGFR inactivation, suggesting a mechanism for persistent colonization in addition to the induction of regulatory T-cells, a hypothesis confirmed *in vivo*, who studied the expression of HBD-2 and HBD-3 in gastric biopsies of infected, uninfected and infection-eradicated healthy subjects. The HBD-2 levels were upregulated in infected subjects and were a marker for gastric mucosal inflammation. In contrast, HBD-3 levels were significantly decreased or unchanged. Eradication therapy led to the normalization of mucosal HBD-2 expression, while HBD-3 expression remained low or showed inter-individual variations.

## 5 Defensins and Respiratory Tract Disease

In the respiratory tract a complex system of defense has evolved that involves structural, physical and functional mechanisms (Whitsett 2002). Ciliated, basal, goblet, brush and small-granule cells, alveolar epithelial, endothelial and interstitial cells, alveolar macrophages, neutrophils, eosinophils, dendritic cells, mast cells, NK and lymphocytes all take part in the innate and adaptive immune system. Ciliated epithelial cells of the nose, trachea and bronchi, and macrophages and type II pneumocytes produce β defensins. HBD-1 is constitutively expressed and acts as a baseline host defense molecule in the absence of injury or inflammation because the transcription factor binding sites are missing. HBD-2 is induced by inflammatory mediators or bacterial products that induce translocation of NF-kB and HBD-2 transcription in

airway epithelial cells (Becker et al. 2000). Kao et al. (2004) showed that IL-17 is the most potent cytokine in stimulating HBD-2 expression in human epithelial respiratory cells. This suggests that the transcriptional regulation of HBD-2 in these cells involves, besides NF-kB or AP-1 (Harder et al. 2000), an IL-17R-dependent JAK signaling pathway. HBD-2 was strongly upregulated in response to proinflammatory stimuli in the epithelium of the respiratory tract (Schutte and McCray 2002). TLR-4 is involved in LPS-induced HBD-2 expression by human A549 epithelial cells (MacRedmond et al. 2005, 2007), leading to the induction of inflammatory mediators and antimicrobial peptides. Tsutsumi-Ishii and Nagaoka (2003) showed that the HBD-2 expression in these cells is amplified by LPS-stimulated monocytes through IL-1β and TNF-α. In the human upper respiratory tract, nasal mucosa (Lee et al. 2002; Kim et al. 2003), larynx (Kutta et al. 2002) and lower respiratory tract (Schaller-Bals et al. 2002), the expression of inducible HBD-2 appears consistently elevated in states of infection. *Pseudomonas aeruginosa* is an opportunistic pathogen that in immunocompromised patients causes severe pulmonary infection with high mortality rates (Crouch Brewer et al. 1996). It is also a major cause of severe chronic pulmonary inflammation in patients with cystic fibrosis (CF), a genetic recessive disorder in which bacterial infection and excessive inflammation induce COPD exacerbation and progressive lung damage (Lieberman 2003). *P. aeruginosa* activates pulmonary A549 epithelial cells through TLR-4 and NF-kB, which regulates some proinflammatory genes, including those encoding IL-8 (Pechkovsky et al. 2000). An increased production of proinflammatory cytokines (CXCL8, TNFα and IL-17) and an elevated number of neutrophils did not enhance bacterial clearance but resulted in lung tissue damage. In cystic fibrosis, due to a genetic defect that results in elevated salt concentrations that inactivate defensins, the antimicrobial peptide defenses fail to provide an effective barrier, resulting in bacterial overgrowth and chronic tissue-destructive inflammation (Smith et al. 1996; Goldman et al. 1997). Little attention has been given to other species of the same genus, such as *P. fluorescens*. This bacterium has long been considered a psychrotrophic microorganism, unable to grow at temperatures over 32 °C. However, in recent years, *P. fluorescens* has been reported to cause blood transfusion-associated septicemia and catheter-related bacteremia among patients with cancer (Gershman et al. 2008) and in nosocomial infections (Chapalain et al. 2008). Donnarumma et al. (2010) and Buommino et al. (2014) showed that some strains isolated from a clinical environment were able to grow at around 37 °C. *P. fluorescens* AF181, isolated from patients with pulmonary tract infections, binds to the surface of cultured A549 human respiratory epithelial cells, involving αvβ5 integrin and its natural ligand vitronectin in adherence and invasion. The strain adheres to A549 cell monolayers with the same efficiency when challenged at 28 °C or 37 °C, and stimulates innate immune responses releasing β defensin 2 and forming a biofilm. While there is a low inflammatory response at 37 °C, inflammatory mediators are induced when the microorganism is grown at a lower temperature. The authors conclude that a low proinflammatory response induced by *P. fluorescens* at 37 °C and the formation of biofilm shows the ability of this microorganism to express specific virulence traits at this temperature and to colonize a susceptible host, thus emerging as an opportunistic pathogen.

## 6 Defensins and Oral Cavity Disease

Antimicrobial peptides are important contributors to maintaining the balance between health and disease in the oral environment. Gingival epithelia are exposed to dental plaque biofilms, and β-defensins expressed in the epithelia, α-defensins in neutrophils, LL-37 expressed both in epithelia and neutrophils exhibit antimicrobial activity against oral microbes including periodontitis-related bacteria, *Candida* and papillomavirus. Histatins are cationic peptides expressed only in salivary

glands (VanderSpek et al. 1989). In patients with reduced salivary flow, as occurs in Sjogren's syndrome, histatin has an important role in the control of oral *Candida* (Jainkittivong et al. 1998). Histatin is active also against azole-resistant *Candida* strains (Situ et al. 2000; Tsai and Bobek 1998). HBD-2 is ten-fold more potent than HBD-1 against periodontopathogenic bacteria. HBD-3 shows a broader spectrum activity than HBD-2 and at a much lower concentration. *S. mutans*, *S. sobrinus*, *S. sanguinis*, *L. acidophilus*, *A. actinomycetemcomitans* and *P. gengivalis* are killed by HBD-3 (Maisetta et al. 2003). In oral epithelium diseases, including oral inflammatory lesions, periodontitis and oral cancer, the secretion of HBDs plays an important role in the protection against oral microbes and in immunomodulation (Signat et al. 2011; Abiko et al. 2007).

## 7 Defensins and Genitourinary Tract Disease

HBD-1 is present in the kidney tissue, loop of Henle, distal tubules of the kidney and collectors, and in the epithelial layers of the vagina, cervix, uterus and fallopian tubes (Valore et al. 1998). In pyelonephritis the concentrations of HBD-1 in urine and plasma are 2–3 times higher than in healthy individuals (Hiratsuka et al. 2000). In prostate and kidney tumors a downregulation of HBD-1 was observed. HBD-2 was induced in the distal tubules in the loops of Henle and collecting ducts in pyelonephritic kidneys (Lehmann et al. 2002). The induced *in-vitro* activity depends on IL-1β, TNF-α, and LPS. α and β-defensins are expressed in the male reproductive tracts of humans, distributed in epididymus and seminiferous tubules, in the germ cells, Leyding cells and Sertoli cells (Yamaguchi et al. 2002) HBD-2 in the genitourinary epithelia protect from infections caused by *Candida* (Wiechuła et al. 2007). In the female reproductive tract, defensins have an important role in fertility and successful pregnancy. HBD-3 expression is very high during the secretory phase of the menstrual cycle. Cytokines such as IL-1β, TNF-α, IFN-γ upregulate *in vitro* the HBD-3 expression in cultures of endometrial epithelial cells. Higher levels of neutrophil α-defensins are detectable in the vagina during infections with *Neisseria gonorrhoeae*, *Trichomonas vaginalis* or *Chlamydia trachomatis* (Donnarumma et al. 2014b). Inflammatory mediators stimulate the induction of HBD-2 in endometrial cells *in vitro* (King et al. 2002). The fetus in utero during the third trimester is covered with a vernix caseosa that contains a collection of antimicrobial peptides particularly active vs Gram-negative bacteria (Yoshio et al. 2003).

## 8 Defensins and Viral Disease

Few studies have examined the role of human defensins in viral diseases. NP-1 inhibits the spread of HSV-2 and the entry and transport of VP16 to the nucleus (Sinha et al. 2003). HBD-2 and HBD-3 inhibit HIV replication by altering the expression of the HIV receptor and coreceptor in PBMCs (Quiñones-Mateu et al. 2003). Besides direct antiviral activity, defensins may also influence innate immunity against viral infections via chemotaxis of immune cells to the site of infection. A direct inactivation of respiratory viruses by defensins has been reported: adenovirus is inactivated by α and β defensins (Bastian and Schäfer 2001), and human rhinovirus (RV-16) induces HBD-2 and HBD-3 in epithelial cells involving the TLR-3 pathway. *In-vivo* HBD-2 levels were increased in nasal lavage (Proud et al. 2004) of subjects infected with human RV. Ryan et al. (2007) showed that plasmacytoid dendritic cells stimulated with influenza virus increase HBD-1 mRNA and this increase is inhibited by ultraviolet inactivation of the virus.

## 9 Defensins and Cancers

Antimicrobial peptides also have an important role in tumor progression or tumor suppression in several malignancies such as oral squamous cell carcinoma (SCC). Scola et al. (2012) studied

the expression of HBD-1, 2 and 3, in actinic keratosis, SCC, healthy skin and chronically ultraviolet-exposed controls. The results obtained show that HBD-1 might act as a tumor suppressor while HBD-2 might act as a promoter of tumor progression. Recently an innovative pathogenetic concept, the so called "immunocompromised cutaneous district" (ICD) was introduced; it refers to a site with an obstacle to normal trafficking of immunocompetent cells through lymphatic channels, or an interference with the signals that the neuropeptides and neurotransmitters released by peripheral nerves send to cell membrane receptors of immunocompetent cells. In these sites there is a propensity for developing a secondary disease, after varying time lapses. Possible causes of ICD are chronic lymphedema, herpetic infection, physical injuries, paraplegia, carpal tunnel syndrome, hemiplegia, or diabetes (Baroni et al. 2011, 2013). A regional immune deregulation has been correlated with ICD. Alterations of innate immunity have been demonstrated, such as a modified gene expression of HBD-2, as well as of TLR-2, desmogleins, MMP-9 and IL-10, but no induction of IL-1α or TNF-α in lymphedematous sites (Baroni et al. 2014). These changes might favor a local appearance or progression of opportunistic diseases such as tumors, infections and immune-mediated skin disorders. An important strategy may be to restore the balance between immunostimulating and immunosuppressive molecules.

## 10 Defensins as a New Class of Therapeutic Drugs

The growing problem of resistance to conventional antibiotics and the need for new antibiotics has greatly stimulated the interest of researchers in the use of antimicrobial peptides for innovative therapeutic strategies. Their significant potential as antimicrobial agents, their role in wound healing, in the modulation of immune responses, angiogenesis, in tissue remodeling, and their ability to bind LPS suggest their use in experimental models of septic shock (Giacometti et al. 2002). Diseases associated with defects in the expression of AMPs continue to be identified. In addition, more and more advanced knowledge on the circuits involved in the regulation of the intracellular expression of AMPs will allow to develop new therapies to suppress or stimulate the expression of these interesting molecules. The intriguing idea of developing natural antimicrobial peptides as innovative antibiotics has been followed up by several biotechnology companies. Several factors, including cost, toxicity, susceptibility to proteases and *in-vitro* folding steps, have limited their use as therapeutic agents (Marr et al. 2006; Hancock and Sahl 2000). Genetic engineering is a strategy to produce large amounts at low cost, but most important is the choice of a system to permit correct folding of the peptide and ensure the effectiveness of the antimicrobial activities (Estrada et al. 2007). Several heterologous systems for producing defensins have been used (Corrales-Garcia et al. 2011), but there is scanty information on the correct folding and biological activities. The application of computational methods to create AMPs with reduced cytotoxicity, reduced proinflammatory potential and reduced salt sensitivity is of great interest. An alternative approach to adding an AMP exogenously is to boost endogenous levels for greater antimicrobial protection and/or immunomodulation. The studies on natural inducers are of increasing interest. The inducible nature of the AMPs suggests the development of new adjuvant therapies to increase the expression of endogenous antimicrobial peptides or proteins for prevention or treatment of diseases. Diseases such as Crohn's disease, atopic dermatitis and some forms of bacterial dysentery may be treatable by administering substances that enhance the expression of specific deficient antimicrobial peptides.

The essential amino acid isoleucine can pharmacologically stimulate β-defensin gene expression in isolated enteric cells, suggesting the development of new classes of safe therapeutic agents (Fehlbaum et al. 2000).

The natural extract of the avocado gratissima fruit (AV119), a patented blend of two rare

sugars, mannoeptulose and perseitol, induces the aggregation of *Malassezia,* inhibits invasiveness in human keratinocytes and sustains a strong HBD-2 response (Donnarumma et al. 2007a). Intracellular signaling pathways and nuclear responses in skin keratinocytes stimulated with AV119 involve the activation of PKC and PTK, which mediate AP-1 activation. The HBD-2 promoter contains NF-kB and AP-1 binding sequences. AV119 leads to a molecular translocation of AP-1 and activates the HBD-2 gene (Paoletti et al. 2010). Interestingly, AV119 is also able to induce a significant increase in HBD-3, which has a broad spectrum of activity against Gram-positive and negative bacteria and fungi and, in particular, protects the skin from colonization by *S. aureus* (Sass et al. 2010). AV119 modulates the HBD-3 expression via TLR-2 and ERK/MAPK phosphorylation in human keratinocytes, as occurs for HBD-2. Thus, AV119 works competitively by inhibiting adhesion and invasiveness of pathogens in keratinocytes and promoting an antimicrobial defense of the host through a TLR-2-dependent HBD-2 and HBD-3 production, but in the absence of a proinflammatory response (Paoletti et al. 2012). These results suggest the use of the sugar in the preparation of cosmetics or pharmacological drugs for the treatment of pathologies associated with a deficit of HBDs.

Dietary supplements such as resveratrol, a natural polyphenol present in the Mediterranean diet and found particularly in grape skin, nuts, pomegranates and *Polygonum cuspidatum*, reduces the incidence of coronary heart disease, cancer and dementia (Frankel et al. 1993; Dorozynski 1997). Resveratrol downregulates endothelins, is antioxidant (Liu et al. 2011), anti-inflammatory and neuroprotective (Albani et al. 2010). Polydatin, the glycoside of resveratrol found in grape juices and in *P. cuspidatum*, is more active than resveratrol. Human keratinocytes treated with resveratrol and polydatin and subsequently heat stressed showed a reduced proinflammatory response, increased levels of HBD-2 and elevated levels of HSP 70B. The authors suggest a rational use of a combination of resveratrol-polydatin in dermocosmetics and pharmacological preparations. This association activates the cytoprotective response, protects the skin from environmental stress (Lanzilli et al. 2012), reduces inflammation and increases the antimicrobial response.

Tacrolimus (FK506), a macrolide isolated from *S. tsukubaensis*, has been used in chronic inflammatory dermatoses for its immunosuppressive effects. The anti-proliferative effects of FK506, correlated with a blockage of the keratinocyte cell cycle at the $G_0$ /$G_1$ phases, explain the therapeutic success in psoriasis. Recently Balato et al. (2014) showed that in keratinocytes infected with *M. furfur*, FK506 did not modify the expression of cytokines (IL-1α, IL-6, IL-8, IL-10, TGF-β1) and did not suppress the enhanced expression of HBD-2 induced by *M. furfur*. These results suggest FK506 as a possible therapeutic option not only for its effects in hyper-proliferative skin disorders, but also for its protective effects regarding infections, in particular mycotic infections that exacerbate skin diseases such as psoriasis or atopic dermatitis. Other studies on small molecule regulators for therapeutic applications in skin diseases have been conducted in recent years. Zinc gluconate is efficient in inflammatory dermatoses, such as acne vulgaris. Poiraud et al. (2012) showed that in an LPS-stimulated skin explant model, pretreatment with zinc gluconate increased the HBD-2 and psoriasin expression levels.

Grether-Beck et al. (2012) in a study on 21 human volunteers showed that topical urea, besides enhancing stratum corneum hydration, regulated the epidermal structure and function in parallel with enhanced antimicrobial peptides, such as HBD-2. They showed that topical urea normalized the barrier function and enhanced AMP expression in a murine model of atopic dermatitis.

Phytochemicals showed a broad spectrum of pharmacological effects including immunostimulatory, antiseptic and anti-inflammatory properties (Hu et al. 2003; Do Monte et al. 2004; Liu et al. 2010). BNO 1030, a herbal medicine product, is an ethanolic-aqueous extract of seven medicinal plants most of which

contain phenolic compounds with anti-inflammatory and anti-oxidative properties. Hostanska et al. (2011) demonstrated that BNO 1030 suppressed the secretion of IL-8 and HBD-2 in cultured epithelial A549 cells. These results support the use of BNO 1030 in diseases of the respiratory tract associated with severe inflammation.

Few studies have examined the possible synergistic interactions among the various antibacterial substances. Antimicrobial peptides have been shown to enhance the potency of antibiotics *in vivo*, probably facilitating the access of the antibiotics to the bacterial cells (Darveau et al. 1991).

Synergistic effects have been demonstrated by histatin-5 and amphotericin B against *Candida* spp., including the amphotericin B-resistant strains, *Cryptococcus neoformans* and *Aspergillus fumigatus* (van't Hof et al. 2000). Combinations of β defensins, LL-37 and tobramycin (an antibiotic used for CF patients by inhalation) were additive.

Besides their antimicrobial effects, several antibiotics were found to have significant immunomodulatory properties both *in vitro* and *in vivo* in an animal model (Stevens 1996).

Fluoroquinolones are broad spectrum antimicrobial agents used for several infections including systemic infections in immunocompromised hosts (Hooper and Wolfson 1991). In Gram-negative infections the use of antibiotics induces the release of LPS with a consequent increase in proinflammatory cytokines. Natural antimicrobial peptides, such as β-defensins, bind LPS and modulate the inflammatory response. Donnarumma et al. (2007b) demonstrated that moxifloxacin (MXF) in combination with HBD-2 induced a marked reduction in proinflammatory cytokines and ICAM-1 in the human lung epithelial cell line A549 stimulated with LPS, compared to treatment with HBD-2 or MXF alone. Thus, the neutralization of LPS proinflammatory effects by MXF/HBD-2, associated with the antimicrobial activity, suggests the advantage of HBD-2 in combination with pharmacological drugs in the therapy of respiratory infections.

In conclusion, the antibacterial potency of the mucosal fluid in the lungs may be increased significantly by synergistic and additive interactions among the antimicrobial factors. Sharma et al. (2000) showed that the combination of HNP-1 with antitubercular drugs (isoniazid and rifampicin) resulted in a significant reduction in the mycobacterial load. Fattorini et al. (2004) showed that the activity of HBD-1 against *M. tuberculosis* is increased by the combination with isoniazid, which reduces the growth of *M. tuberculosis* compared to the peptide or isoniazid alone, due to increased permeability of the *Mycobacterium* cell wall and cell membrane by HBD-1.

The bactericidal activity of HBD-3 on *Actinobacillus actinomycetemcomitans*, *P. gingivalis* and *S. mutans* was evaluated in combination with lysozyme, amoxycillin, metronidazole or chlorhexidine (Maisetta et al. 2003). The combination of a non-bactericidal concentration of HBD-3 and amoxycillin, chlorhexidine and metronidazole killed *A. actinomycetemcomitans* after 1 h of incubation. Similar effects were obtained with the combination of HBD-3 with amoxycillin and chlorhexidine against *P. gingivalis*. Enhanced bactericidal effects were observed in a combination of HBD-3 with metronidazole against *P. gingivalis* after 1.5 h and with amoxycillin, chlorhexidine and lysozyme against *S. mutans*. The authors concluded that the effects are potentiated by the combination of drugs because the antibiotics damage the cell wall (amoxycillin) or outer membrane (chlorhexidine) and facilitate the penetration of HBD-3, which, by increasing the permeability of the outer or inner membranes, facilitates the entry of the antibiotics.

## 11 Conclusions

Antimicrobial peptides have provided multicellular organisms with defenses needed to live in environments dominated by microbes. Their antimicrobial activity has been shown against bacteria, fungi, viruses and protozoa.

The increase in the resistance of bacterial to conventional antibiotics and the need for new antibiotics has stimulated interest in the use of AMPs as a new therapeutic agent. The inducible nature of many antimicrobial peptide genes suggests that it is possible to increase the endogenous production by utilizing small molecules of various origins to enhance the expression of these peptides, even selectively. In addition, after the discovery of an intracellular circuit involved in the regulation of the expression of antimicrobial peptides, new generations of therapeutics will be developed to suppress or stimulate the expression of AMPs.

In the light of the different activity of AMPs, such as their role in immunomodulation, angiogenesis, wound healing, inflammation, cancer, as well as their antimicrobial activity, it is possible induce the expression of specific antimicrobial peptides or design analogs with an increased specific activity or various degrees of selectivity, or obtain AMPs with genetic engineering to optimize both the potency and safety by reducing cytotoxicity and potential proinflammatory effects and susceptibility to protease and salt. Antimicrobial peptides have been shown to enhance the potency of antibiotics *in vivo* by facilitating their access into the bacterial cells and through the neutralization of LPS. Restoring the balance between immunostimulating and immunosuppressive molecules may be an important strategy to correct expression defects present in specific diseases.

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# Emergence of *Escherichia coli* Sequence Type 131 (ST131) and ST3948 with KPC-2, KPC-3 and KPC-8 carbapenemases from a Long-Term Care and Rehabilitation Facility (LTCRF) in Northern Italy

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**Abstract**

Aim of the study was to characterize KPC-producing *Escherichia coli* (KPC-Ec) clinical isolates among a Northern Italy Long-Term Care and Rehabilitation Facility (LTCRF) residents. Thirteen consecutive non repeated MDR *E. coli* isolates showing ertapenem Minimum Inhibitory Concentrations (MICs) >0.5 mg/L, collected during the period March 2011 – May 2013 from ASP "Redaelli" inpatients, were investigated. The $bla_{\text{KPC/CTX-M/SHV/TEM/OXA}}$ genes were identified by PCR and sequencing. KPC-Ec isolates underwent phylotyping, Pulsed-Field Gel Electrophoresis (PFGE), multilocus sequence typing (MLST) and repetitive sequence-based PCR (rep-PCR) profiling. Incompatibility groups analysis and conjugation were also performed. Eleven out of 13 isolates, resulted $bla_{\text{KPC}}$-type positive, were consistently resistant to third generation cephalosporins, fluoroquinolones and trimethoprim-sulphametoxazole (84.6 %), retaining susceptibility to colistin (EUCAST guidelines). At least n = 4/11 of KPC-Ec patients received ≥48 h of meropenem therapy. Sequencing identified 9 $bla_{\text{KPC-2}}$, 1 $bla_{\text{KPC-3}}$ and 1 $bla_{\text{KPC-8}}$ determinants. KPC-Ec plasmids belonged to IncF group (FIIk replicon); conjugation confirmed $bla_{\text{KPC/TEM-1/OXA-9}}$ genes transferability

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for 10 KPC-Ec. Although three pulsotypes (A, B, C) were identified, all KPC-Ec belonged to phylogenetic group B2. Clone B (B-B5) caused an outbreak of infection involving nine inpatients at five wards. Rep-PCR showed relatedness for seven representative KPC-Ec isolates. Here we report a LTCRF outbreak caused by a ST131-B2 *E. coli* associated with $bla_{KPC-2}$ and $bla_{KPC-8}$ genes, and the emergence of the new ST3948. Elderly people with co-morbidities are at risk for ST131 colonization. KPC-Ec clones local monitoring appears essential both to avoid their spreading among healthcare settings, and to improve therapeutic choices for LTCRF residents.

**Keywords**

Long-Term Care and Rehabilitation Facility • KPC-positive *E. coli* • Sequence Type 131 (ST131) • ST3948

## 1 Introduction

Carbapenemase-producing *Enterobacteriaceae* (CPE) isolates have been increasingly reported in Europe (Grundmann et al. 2010; Miriagou et al. 2010). The results of the last countrywide cross-sectional survey, carried out during 2011 to investigate the diffusion of Carbapenem-Resistant *Enterobacteriaceae* (CRE) in Italy, showed that CRE were 2 % of all collected isolates, *Klebsiella pneumoniae* being the most frequent species (87 %). Carbapenemase production was the main mechanism (85 %) involved, with $bla_{KPC}$ gene detected mainly in *K. pneumoniae* and only in one *Escherichia coli* CRE (Giani et al. 2013). While less prevalent than in *K. pneumoniae*, KPC production in other species of *Enterobacteriaceae* is increasingly reported, presumably as $bla_{KPC}$-carrying plasmids are acquired from *K. pneumoniae* by these non-*Klebsiella* species and then propagate (O'Hara et al. 2014). After the first detection of a KPC-positive *E. coli* (KPC-Ec) in Europe, reported in 2008 in France from a patient initially hospitalized in Israel (Petrella et al. 2008), sporadic isolation of KPC-Ec strains has been reported in the USA (Urban et al. 2008), Israel (Goren et al. 2010) and European countries (Naas et al. 2011; Morris et al. 2011). Moreover, *in vivo* transfer of KPC-2 and KPC-3 from *K. pneumoniae* to *E. coli* of different Sequence Types (STs) as ST131, ST1672 and ST394 has been recently described in Italy (Richter et al. 2011; Gona et al. 2014).

*E. coli* ST131, designated according to the Achtman multilocus sequence typing (MLST) system, was identified in 2008 as a major clone related to the spread of the CTX-M-15 Extended-Spectrum β-lactamase (ESβL) and became the single most prevalent human extra-intestinal *E. coli* strain in many regions, especially among fluoroquinolone- and/or Extended Spectrum-cephalosporin-resistant isolates (Coque et al. 2008; Nicolas-Chanoine et al. 2014). The increasing detection of ST131 isolates from hospitalized and non-hospitalized individuals and, more recently, from companion (Pomba et al. 2014) and foodborne animals (Ghodousi et al. 2015; Platell et al. 2011), sewage and main rivers of large European cities (Colomer-Lluch et al. 2013), highlights the rapid spread and local adaptation to different habitats of this lineage. Worryingly, strains of *E. coli* ST131 resistant to carbapenems have also been reported, further limiting treatment options for this clone (Petrella et al. 2008; Urban et al. 2008; Goren et al. 2010; Naas et al. 2011; Morris et al. 2011). Sporadic isolations of KPC-Ec strains have been reported from Ireland (Morris et al. 2011), France (Naas et al. 2011),

Italy (Accogli et al. 2014), United States (Kim et al. 2012), Taiwan (Ma et al. 2013) and China (Cai et al. 2014).

Since Long-Term Care and Rehabilitation Facilities (LTCRFs), essential components of healthcare delivery to many patients, have been recently recognized as "reservoirs of antibiotic resistance" (Viau et al. 2012) and the spread of carbapenemase-producer *E. coli* strains is related to certain pandemic clones (ST131); aim of the study was: (i) to investigate the presence of KPC-Ec in an Italian LTCRF, (ii) describe KPC-Ec molecular and epidemiological features.

## 2 Materials and Methods

### 2.1 Setting

The Clinical Microbiology Laboratory of the ASP "Golgi-Redaelli" of Milan collects and analyzes biological samples from three different Geriatric Institutes: the "P. Redaelli" of Vimodrone, (n = 308 beds), the "C. Golgi" of Abbiategrasso (n = 334 beds) and finally the "P. Redaelli" of Milan (n = 310 beds).

### 2.2 Bacterial Strains

Thirteen clinical consecutive non-replicate isolates of *E. coli* with ertapenem (ETP) MIC >0.5 mg/L by Phoenix System (Becton Dickinson Diagnostic Systems, Sparks, USA) were collected in the period from March 2011 to May 2013 at the ASP "Golgi-Redaelli", in Milan. All the *E. coli* isolates were obtained from infected elderly inpatients at different long term and rehabilitation Vimodrone wards. Strains were mainly from urine (n = 11/13; 84.6 %) and in two cases from sputum specimens. Among urine samples, 5/11 were from short-term and the remaining six from indwelling catheters. Demographic, clinical and antibiotic administration data were collected through medical records.

### 2.3 Antimicrobial Susceptibilities, Detection of Carbapenemases and Other β-Lactamases

Identification and susceptibility profiles were confirmed using MicroScan4 (Beckman Coulter) NBC46 panels. MICs of imipenem (IPM), meropenem (MER) and ETP were obtained by Etest strips (bioMérieux). Results were interpreted according to EUCAST guidelines (http://eucast.org/clinical_breakpoints). *E. coli* ATCC 25922 was used as a quality control strain. The isolates were screened for their ability to produce carbapenemases by Modified Hodge Test (MHT) and by the confirmatory disk test using ETP and ETP plus aminophenylboronic acid (APBA) or EDTA. Detection of $bla_{\text{SHV-}}$, $bla_{\text{TEM-}}$, $bla_{\text{CTX-M-}}$, $bla_{\text{OXA-}}$ and $bla_{\text{KPC}}$-type genes was performed by PCR as previously described (Tzelepi et al. 2003; Giakkoupi et al. 2009). PCR amplicons were purified using the kit Quantum Prep PCR Kleen Spin Columns (Bio-Rad) and subjected to direct sequencing. PCR products were sequenced on both strands with an Applied Biosystems sequencer. The nucleotide sequences were analyzed with the BLAST software program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

### 2.4 Isoelectric Focusing

Production of β-lactamases was confirmed by analytic isoelectric focusing (IEF). IEF of crude cell extracts allowed the visualization of β-lactamase bands by nitrocefin (gels were electrophoresed at 11–14 W for 90 min and β-lactamase bands were detected using 0.5 mM nitrocefin). The β-lactamase bands activity was assessed by a substrate overlaying procedure (using 0.5 μg/ml ETP) as previously described (Pagani et al. 2002). Crude enzymatic extracts from well-known β-lactamase producers (i.e., TEM-1, TEM-2, TEM-7, TEM-8, TEM-9, TEM-12, SHV-1, SHV-2 and SHV-5) were used as controls.

## 2.5 Conjugation Experiments and Plasmid Characterization

Conjugative transfer of plasmids was carried out in mixed broth cultures using *E. coli* K12 strain J62 ($F^-$, *pro*, *his*, *trp*, *lac*, $Sm^r$) and J53 ($F^-$, *met*, *pro*, $Rif^r$) as recipient strains. *E. coli* transconjugants were selected on McConkey agar containing ETP (0.5 mg/L) and streptomycin (1000 mg/L) or rifampin (100 mg/L), respectively. Colonies grown on the selective medium were picked for identification by the MicroScan4 System. Recipients that harbored $bla_{KPC}$-type genes and exhibited resistance to carbapenems and cephalosporins were defined as transconjugants.

Plasmid DNA of *E. coli* isolates and transconjugants were obtained using Pure Link HiPure Plasmid Midiprep kit (Invitrogen, by Life Technologies) and were separated by electrophoresis. Plasmid incompatibility groups were determined in both donor and transconjugant strains by the PCR-based replicon typing (PBRT) method (Carattoli et al. 2005) using the commercially available PBRT Kit (Diatheva) according to manufacturer's instructions.

## 2.6 PFGE and Rep-PCR Analysis

PFGE was performed using *Xba*I restriction enzyme and fragments were separated on a CHEF-DR II apparatus (Bio-Rad, Milan, Italy) for 22 h at 14 °C. Bacteriophage λ concatenamers were used as DNA size markers. DNA restriction patterns of scanned gel pictures were interpreted following cluster analysis with the Fingerprinting II version 3.0 software (Bio-Rad) using the unweighted pair-group method with arithmetic averages (UPGMA). Only bands larger than 48 kb were considered for the analysis. The Dice correlation coefficient was used with a 1.0 % position tolerance to analyze the similarities of the banding patterns, and a similarity threshold of 90 % to define clusters. The restriction patterns of the genomic DNA from the isolates were analysed and interpreted according to the criteria of Tenover et al. (1995), too.

Repetitive sequence-based PCR (rep-PCR) was performed with the semiautomated Diversilab system (DL) (bioMérieux), according to the manufacturer's instructions. DNA extraction was performed with the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories Inc). Analyses of the PCR amplicons were performed using a 2100 Bioanalyzer (Agilent Technologies). DL fingerprints were analyzed with the DL software 3.4, using the Pearson correlation statistical method to determine clonal relationships. For isolates to be considered indistinguishable in addition to >97 % similarity no peak differences should be seen. For similar isolates, in addition to 95–97 % similarity one to two peak differences are accepted. In addition to <95 % similarity more than two peak variations are considered different. In this study we used the criteria for "indistinguishable" for defining DL profiles (Brolund et al. 2010).

## 2.7 MLST and Phylogenetic Typing

MLST of representative *E. coli* isolates was performed according to the protocol of Wirth et al. (2006). Allelic profiling and sequence type (ST) determination were performed using the *E. coli* MLST scheme from the website of the University of Warwick (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

Phylogenetic groups were determined by a two-step triplex PCR described by Clermont et al. (2000) using Multiplex PCR kit (Qiagen).

## 2.8 Demographic and Clinical Data

Clinical records of patients with KPC-Ec infection or colonization were examined retrospectively. The following data were recorded: age, gender, admission ward, date of admission, previous hospitalization during the last year, site of infection or colonization, antimicrobial administration before or during the infective episode, treatment outcome.

Treatment failure was classified as absence of resolution or worsening of signs and symptoms

of infection; not assessable – due to incomplete records of the patient within 72 h of infection.

## 3 Results

### 3.1 Antimicrobial Susceptibilities and $bla_{KPC}$ Genes Detection

A total of 13 consecutive, non-duplicate *E. coli* isolates showing ETP >0.5 mg/L MIC (0.06 % of the strains of the same species processed in that period) were identified at Clinical Microbiology Laboratory of ASP "Golgi-Redaelli". Susceptibility results initially obtained by Phoenix System (BD) were then confirmed at the Pavia University by both MicroScan4 System (Beckman Coulter) and Etest (bioMérieux).

The isolates showed a MIC range for ETP of 1 to >32 mg/L ($MIC_{50}$ = 4 mg/L; $MIC_{90}$= >32 mg/L), MER of 0.064 to >32 mg/L ($MIC_{50}$ = 0.5 mg/L; $MIC_{90}$ = 4 mg/L) and IPM of 0.5 to >32 mg/L ($MIC_{50}$ = 1 mg/L; $MIC_{90}$ = 4 mg/L) (Table 1). All the isolates were resistant to piperacillin, amoxicillin/clavulanate, third generation cephalosporins, aztreonam, and ciprofloxacin; 11/13 (84.6 %) strains were resistant to trimethoprim-sulphametoxazole (SXT) and 2/13 (15.4 %) to amikacin. All isolates retained susceptibility to colistin and tigecyclin.

Although the resistance phenotypes of all the 13 isolates were suggestive of a carbapenemase production, the results of both the MHT and the confirmatory disk test using ETP and ETP + APBA were positive only for 11/13 isolates (84,6 %), that underwent molecular typing and were examined for the presence of $bla_{KPC}$-type genes. The remaining two *E. coli* strains resulted negative to both modified Hodge and ETP-EDTA disk combination tests.

Screening for $bla_{KPC}$ determinants by PCR yielded an amplification product of the expected size from the same 11/13 isolates. Sequencing of the above 11 $bla_{KPC}$ positive isolates, identified the resistance gene as $bla_{KPC-2}$ (n = 9) or $bla_{KPC-3}$ (n = 1) and $bla_{KPC-8}$ (n = 1) (Table 1).

Analytical IEF of crude extracts of all the *E. coli* clinical isolates revealed heterogeneous patterns, with multiple β-lactamase bands in 10/13 cases. The presence for 11/13 samples of a pI 6.7 band showing activity on ETP in a bioassay, was consistent with the production of the KPC enzymes. Such a band was accompanied in 10/11 cases by other two bands (pI 7.2 and pI 5.4) the first able to hydrolyse oxacillin, and the other one showing a narrow spectrum of activity by bioassay. Only a pI >8.2 band, showing high level of hydrolytic activity on CTX (1 mg/L) and consistent with the production of an ESBL of CTX-M-type was present in 2/13 isolates. The BL types detected by PCR in the KPC-Ec included OXA-9 for all isolates and TEM-1 in 10/11 cases. The $bla_{CTX-M}$-type gene presence was confirmed by PCR in the two non-KPC producers; none of the isolates studied was positive for the presence of $bla_{SHV}$-type genes.

### 3.2 Molecular Characterization and Typing

The plasmids replicon typing highlighted the presence of two plasmids belonging to the incompatibility group IncF (FIIk replicon and about 200 Kb in size) and IncF multireplicon (FII, FIA and FIB), often associated with the $bla_{KPC}$ gene dissemination. Conjugation results highlighted the transferability of both plasmids.

All the 11 KPC-Ec strains belonged to the phylogenetic group B2, and showed multiclonal pulsotypes after *XbaI* digestion. PFGE results showed the presence of three KPC-Ec different clones named A, B, and C. Clone B sub-types, named from B to B5 in order of appearance, are shown in Fig. 1a. Clone A appeared once in April 2011; Clone B emerged on June 2012 at Vimodrone LTCRF and persisted until April 2013 causing an intra-hospital outbreak of infection involving nine inpatients at five different wards (V3/1, V4/1, V3/5, VR1, V4/5). The clone C was obtained on May 2013.

Six KPC-Ec isolates, chosen as representative of both KPC variants and PFGE clones, were furthermore investigated by MLST. Two different STs were overall found: the hypervirulent ST131 and the single locus variant in *purA* allele

**Table 1** Characteristics of the 13 *E. coli* isolates considered in the study

| ID | Collection date (yyyy/mm/dd) | MicroScan4 MIC, mg/L (Susceptibility category) | | | Etest MIC, mg/L (Susceptibility category) | | | Beta-Lactamase (BL) content | | Molecular typing | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IPM | MER | ETP | IPM | MER | ETP | Carbapenemase | BL | PFGE | MLST | DL | Phylogenetic group |
| VR | 2011-03-09 | <=1 (S) | <=1 (S) | 1 (I) | 0.25 (S) | 0.064 (S) | 4 (R) | – | CTX-M Gr. 1 | – | | | D |
| ZG | 2011-04-08 | 4 (I) | 8 (I) | >1 (R) | 1 (S) | >32 (R) | 8 (R) | KPC-2 | OXA-9 | A | 131 | A | B2 |
| RA | 2011-09-30 | <=1 (S) | <=1 (S) | >1 (R) | 0.75 (S) | 0.125 (S) | 3 (R) | – | CTX-M Gr. 2 | – | | | B2 |
| NE | 2012-06-20 | 8 (I) | 8 (I) | >1 (R) | 2 (S) | 0.5 (S) | 1,5 (R) | KPC-2 | TEM-1; OXA-9 | B | 131 | A | B2 |
| PA | 2012-07-03 | <=1 (S) | <=1 (S) | >1 (R) | 0.5 (S) | 0.5 (S) | 1,5 (R) | KPC-2 | TEM-1; OXA-9 | B | | | B2 |
| GE | 2012-10-02 | >8 (R) | 8 (I) | >1 (R) | 1 (S) | 1 (S) | >32 (R) | KPC-2 | TEM-1; OXA-9 | B2 | 131 | A | B2 |
| SM | 2012-10-15 | >8 (R) | >8 (R) | >1 (R) | 1 (S) | 0.38 (S) | 4 (R) | KPC-2 | TEM-1; OXA-9 | B1 | 131 | A | B2 |
| BE | 2012-12-03 | 4 (I) | 8 (I) | >1 (R) | 0.5 (S) | 1 (S) | 2 (R) | KPC-2 | TEM-1; OXA-9 | B3 | | | B2 |
| RMC | 2012-12-05 | <=1 (S) | <=1 (S) | 2 (R) | 1 (S) | 0.5 (S) | 2 (R) | KPC-2 | TEM-1; OXA-9 | B2 | 131 | A | B2 |
| SS | 2013-02-15 | <=1 (S) | <=1 (S) | >1 (R) | 4 (I) | 1,5 (S) | 24 (R) | KPC-2 | TEM-1; OXA-9 | B4 | | | B2 |
| PS | 2013-02-15 | <=1 (S) | <=1 (S) | >1 (R) | 1 (S) | 0.25 (S) | 1 (I) | KPC-8 | TEM-1; OXA-9 | B | 131 | A | B2 |
| DFG | 2013-04-05 | <=1 (S) | <=1 (S) | >1 (R) | 4 (I) | 4 (I) | >32 (R) | KPC-2 | TEM-1; OXA-9 | B5 | | | B2 |
| MD | 2013-05-06 | 32 (R) | 32 (R) | 32 (R) | 2 (S) | 1 (S) | >32 (R) | KPC-3 | TEM-1; OXA-9 | C | 3948 | A | B2 |

*S* susceptible, *I* intermediate, *R* resistant, *IPM* imipenem, *MER* meropenem, *ETP* ertapenem, *PFGE* pulsed-field gel electrophoresis, *MLST* multilocus sequence typing, *DL* Diversilab

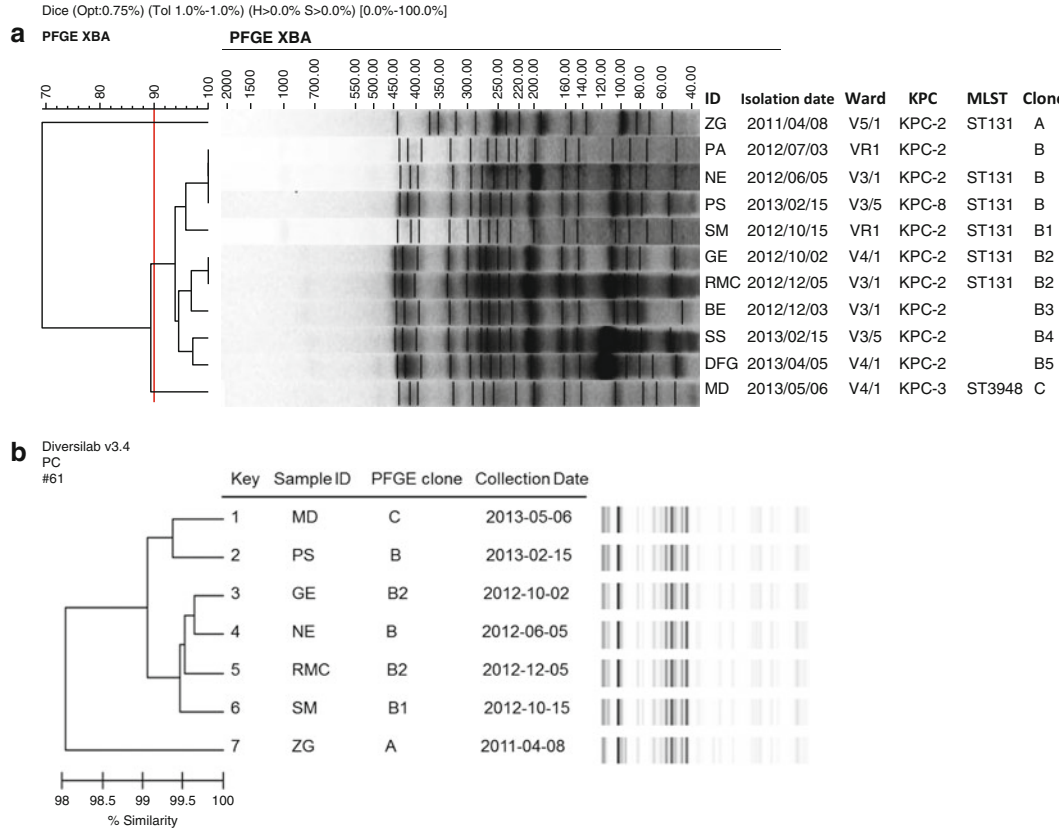


**Fig. 1** (**a**) UPGMA dendrogram of *Xba*I PFGE profiles of KPC-Ec isolates. (**b**) Gel image and dendrogram of rep-PCR DL patterns of KPC-Ec representative strains

(53-40-47-13-36-**10**-29) ST3948 clone, belonging to the ST131 Clonal complex (Clpx). The ST131 KPC-Ec group was identified in five out of six KPC-Ec chosen as representative. Interestingly, pandemic ST131 included both the PFGE clones A and B, the latter group of KPC-Ec strains harboring both the $bla_{\text{KPC-2}}$ or the $bla_{\text{KPC-8}}$ gene variants.

The ST3948 clone was associated to the $bla_{\text{KPC-3}}$ determinant and to the PFGE unique clone C strain.

## 3.3 DL and PFGE

Rep-PCR (DL) on seven representative KPC-Ec isolates, belonging to A, B, C PFGE clones, showed genetic relatedness (Fig. 1b). While ST3948 was included in a distinctive clone C by PFGE, ST131 strains clustered >97 % with ST3948 using the DL tool.

## 3.4 Demographic and Clinical Data of KPC-Ec Patients

According to clinical records, the mean age of the patients was 85 years, with the 54.5 % being female. Based on clinical data, at least n = 4/11 of KPC-Ec infected patients received ≥48 h of MER therapy in an Acute Care Hospital (ACH) and/or LTCRF located in the area of Milan.

All the patients were affected by several co-morbidities, including diabetes, hypertension, chronic obstructive pulmonary disease. Co-infections were reported for the majority of patients: 5/9 caused by *Clostridium difficile,* 1/9 by *Staphylococcus aureus* and in the remaining

cases by Gram-negative bacteria (Table 2). It is of note that three out of 11 patients died during hospitalization. Death was attributable or related to KPC-Ec at least in the outbreak index patient NE.

Epidemiological analysis showed that index patient of the KPC-Ec outbreak, was a 89 years old woman (NE), admitted in April 2012 to the V3/1 rehabilitation word of the "P. Redaelli" LTCRF of Vimodrone. The patient was discharged from the S. Raffaele Hospital of Milan just before. After 2 months of hospitalization, KPC-Ec strain was isolated from the urine of the patient. On admission, patient's physical examination revealed decubitus ulcers and gastroenteritis symptoms. She was treated with metronidazole 500 mg and ciprofloxacin 400 mg. Due to the occurrence of UTI-related septic shock symptoms, a combination therapy with MER and amikacin (500 mg each) started from May 2012 the 5th. A carbapenem resistant *K. pneumoniae* isolate was obtained from the same patient in May 2012 the 9th. The *K. pneumoniae* strain resulted $bla_{\text{KPC-}}$, $bla_{\text{TEM-}}$, $bla_{\text{OXA}}$-type genes producer, and harboured an IncF (FIIk replicon) plasmid, like the KPC-Ec isolate (data not shown).

Due to NE KPC-Ec isolation (one month later) an 8 days colistin plus IPM therapy was administrated; even so, on June the 20th the rectal swab sampling revealed the intestinal persistence of the same strain.

During the next year, eight additional inpatients at the same LTCRF, affected by UTIs (n = 7) and pneumonia (n = 1), resulted positive for the presence of clone B outbreak strain.

The mainly used antibacterial agents are listed in Table 2.

A complete or partial response was obtained in eight patients, whereas failure of treatment or relapse was observed in three patients.

## 4 Discussion

The present study describes the emergence and intra-hospital spread of a clonal strain KPC-Ec in one LTCRF hospital located in Northern Italy and admitting patients from several ACH of the same area. Genetic features and molecular epidemiology were also investigated.

Although KPC-*K. pneumoniae* (KPC-Kp) has become endemic in Italy in many ACH, the presence of KPC-Ec isolates remain limited (Giani et al. 2013). The spread of $bla_{\text{KPC}}$-type resistance determinants is of particular concern in *E. coli*, being emerging KPC-Ec associated with different STs (Mavroidi et al. 2012; Almeida et al. 2012; Baraniak et al. 2011; Ruiz-Garbajosa et al. 2013; Gijón et al. 2012). Even more worrisome is the occurrence and dissemination of the MDR ST131 KPC-Ec clones, belonging to the hypervirulent, uropathogenic lineage B2 (Clermont et al. 2009), and distinguishable in turn into several pulsotypes (Coque et al. 2008; Lau et al. 2008; Nicolas-Chanoine et al. 2013).

In the present study, we report the presence of 11 KPC-Ec producers in an Italian LTCRF.

Interestingly, Etest MIC values for IPM (from 0.5 to 4 mg/L) and MER (from 0.5 to >32 mg/L) resulted within the susceptible clinical breakpoints for the majority of the carbapenemase producers (n = 9/11). Such variability, here observed also in the case of ETP (MIC = 1 – >32 mg/L), was already reported for KPC-Ec strains (Accogli et al. 2014; Deshpande et al. 2006). Moreover, the diversity in carbapenem MICs among KPC-Ec strains of the same clone (B-B5) could be due to differences in plasmid copy numbers (as a result of antibiotic therapy administered) or the presence of sub-populations expressing different resistance levels. Scattered colonies within the Etest inhibition zone were present in few cases, and ETP (instead of MER) resulted the most efficient and sensitive molecule for KPC-Ec producers detection.

A porin loss mechanism coupled with the CTX-M-type enzyme production could justify the ETP MIC values obtained for two non-carbapenemase producers by Microscan4 System (1–>1 mg/L) *versus* Etest (4–3 mg/L).

Overall, phenotypical tests, IEF, PCR and sequencing revealed heterogeneous β-lactamase patterns: while in the case of the two MDR

**Table 2** Clinical and epidemiological data of KPC-Ec infected patients

| Patient | | Admission ward | Admission date (yyyy/mm/dd) | Previous hospitalization | Previous exposure to CB | Reason for admission | Sample | KPC-Ec isolation date (yyyy/mm/dd) | Other bacteria | Therapy | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | Age/Sex | | | | | | | | | Agent | Duration (days) |
| ZG | 77/F | V3/1 | 2011/02/26 | Yes (Policlinico, MI) | NA | Ictus, COPD, diabetes, decubitus, AH, pneumonia | Urine (IC) | 2011/04/08 | – | SAM | 10 |
| | | | | | | | | | | MER | 5 |
| [a]NE | 89/F | V3/1 | 2012/04/02 | Yes (SRH, MI) | Yes (MER) | Decubitus ulcer, gastroenteritis, AH, UTI | Urine (IC) | 2012/06/05 | *K. pneumoniae* | MDZ | 10 |
| | | | | | | | | | *P. aeruginosa* | CIP | 10 |
| | | | | | | | | | | AK | 8 |
| | | | | | | | | | | CO | 8 |
| | | | | | | | | | | IPM | 8 |
| | | | | | | | | | | VA | 8 |
| | | | | | | | | | | AMC | 10 |
| [a]PA | 92/F | VR1 | 2012/05/09 | Yes (SRH, MI) | No | Femur fracture, AH, decubitus, pneumonia | Urine (IC) | 2012/07/03 | *C. difficile* | LEV | 10 |
| | | V4/1 | | | | | | | | VA | 11 |
| | | | | | | | | | | CAZ | 7 |
| | | | | | | | | | | Cefuroxim | 10 |
| | | | | | | | | | | MER | 10 |
| | | | | | | | | | | CRO | 5 |
| GE | 85/M | V4/1 | 2012/09/12 | Yes (Melegnano, MI) | No | COPD, hydrocephalous, diabetes, UTI | Urine (STC) | 2012/10/02 | – | TZP | NA |
| | | | | | | | | | | CO | 7 |
| SM | 85/M | VR1 | 2012/09/19 | Yes (SRH, MI) | Yes (MER) | COPD, pneumonia, UTI, cerebral haemorrhage | Urine (IC) | 2012/10/15 | *P. mirabilis* | CRO | 5 |
| | | V4/5 | | | | | | | *K. pneumoniae* | TZP | 5 |
| | | | | | | | | | | IPM | 5 |
| | | | | | | | | | | LEV | 8 |
| | | | | | | | | | | FOS | 10 |
| | | | | | | | | | | CIP | 7 |
| BE | 87/F | V3/1 | NA | Yes (G. Pini, MI) | No | Pelvis fracture, COPD, AH | Urine (STC) | 2012/12/03 | *K. pneumoniae* | CIP | 10 |
| | | | | | | | | | | FOS | 3 |
| | | | | | | | | | | NFR | 10 |

(continued)

**Table 2** (continued)

| Patient | | Admission ward | Admission date (yyyy/mm/dd) | Previous hospitalization | Previous exposure to CB | Reason for admission | Sample | KPC-Ec isolation date (yyyy/mm/dd) | Other bacteria | Therapy | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | Age/Sex | | | | | | | | | Agent | Duration (days) |
| RMC | 84/F | V3/1 | 2012/11/16 | Yes (SRH, MI, Melegnano, MI) | | Cerebral hematoma, pneumonia | Urine (IC) | 2012/12/05 | *C. difficile* | TZP | 5 |
| | | | | | | | | | | VA | 4 |
| SS | 87/F | V3/5 | 2013/02/11 | Yes (SRH, MI) | No | Femur fracture, AH, diabetes, UTI | Urine (STC) | 2013/02/15 | *P. mirabilis* | NFR | 3 |
| | | | | | | | | | *C. difficile* | AK | 6 |
| | | | | | | | | | | AMC | 10 |
| | | | | | | | | | | MER | 10 |
| PS | 85/M | V3/5 | 2013/01/09 | Yes (Melegnano, MI SRH, MI) | No | Femur fracture, COPD, AH, diabetes, pneumonia | Urine (STC) | 2013/02/15 | *C. difficile* | LEV | 5 |
| | | | | | | | | | | Metronidazole | 3 |
| | | | | | | | | | | CAZ | 3 |
| | | | | | | | | | | NFR | 3 |
| | | | | | | | | | | VA | 15 |
| [a]DFG | 82/M | V4/1 | 2012/11/29 | No | Yes (MER) | COPD, diabetes, AH | Sputum | 2013/04/05 | *C. difficile* | Metronidazole | 3 |
| | | | | | | | | | | CAZ | 12 |
| | | | | | | | | | | AK | 15 |
| | | | | | | | | | | VA | 35 |
| MD | 84/M | V4/1 | 2013/02/27 | Yes (Cernusco, MI, SRH, MI) | Yes (MER) | Femur fracture, COPD, pneumonia | Sputum | 2013/05/06 | *S. aureus* | CAZ | 15 |
| | | | | | | | | | | Zetamicin | 15 |
| | | | | | | | | | | TZP | 7 |

*CB* carbapenems, *SRH* S. Raffaele Hospital, *COPD* chronic obstructive pulmonary disease, *AH* aortic hypertension, *IC* indwelling catheter, *STC* short-term catheter, *MDZ* metronidazole, *CIP* ciprofloxacin, *CAZ* ceftazidime, *MER* meropenem, *AK* amikacin, *CO* colistin, *IPM* imipenem, *VA* vancomycin, *CRO* ceftriaxone, *FOS* fosfomycin, *SAM* ampicillin–sulbactam, *AMC* amoxicillin-clavulanate, *NFR* nitrofurantoin, *NA* not available

[a]Patients with an adverse outcome

KPC-Ec negative isolates only a CTX-M-type ESBL was detected, KPC-2, KPC-3 and KPC-8 MDR strains were also OXA-9 and TEM-1 enzymes producers. Conjugation experiments results together with the plasmid replicon typing analysis (incompatibility group IncF, with FIIk replicon, typical of *K. pneumoniae*) suggested the possible inter-species plasmid exchange between KPC-Kp and KPC-Ec in the retrospectively ascertained (NE) index case patient. Since the clone B strain harbored the 200Kb outbreak conjugative plasmid, the $bla_{KPC-8}$ gene could be due to two points mutations within the $bla_{KPC-2}$ gene sequence (T to G at position 716 leading to a valine-to-glycine substitution, and C to T at 814 position leading to an histidine-to-tyrosine substitution) (data not shown). Nine residents and five wards were involved in the Vimodrone outbreak and the time elapsed between admission and outbreak strain acquisition was in average 38 days.

The molecular and epidemiological results revealed the intra-hospital spread of a ST131-B2 (PFGE clone B) outbreak clone. To note that PFGE clone B-related isolates clustered with two KPC-Ec strains collected on August and October 2012 at Vimercate ACH (our personal data). This confirms how elderly people with many co-morbidities are at greatest risk for ST131 colonization, possibly due to healthcare-associated transmission.

Here we describe the emergence in an Italian LTCRF of three KPC-Ec PFGE clones (A, B, C), belonging to ST131 and to the new ST3948 (clone C). Although LTCRFs are well known as reservoirs of other antimicrobial resistant pathogens (Arnoldo et al. 2013) and have been implicated as reservoirs of ST131 in Europe (Burke et al. 2012; Brisse et al. 2012), at our knowledge this study is the first one describing an Italian LTCRF as KPC-Ec ST131 reservoir. DL results clustered all the KPC-Ec isolates in the same profile, thus confirming the higher discriminatory power of PFGE for *E. coli* outbreak studies.

Taken together, our findings highlight the need to continue screening of *E. coli* showing a decreased susceptibility to ETP, and to implement more rigorous infection control measures among LTCRF residents to avoid the spread of difficult to treat ST131 Clpx KPC-Ec in our area. Failure of treatment was observed in three patients, of which two infected by an KPC-Ec strain studied: local monitoring of KPC-Ec clones appears essential to avoid the risk of their spreading among healthcare settings and to improve effective and appropriate therapeutic choices for LTCRF residents.

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# Carbapenem-Resistant *Klebsiella pneumoniae*: Results of a Laboratory Surveillance Program in an Italian General Hospital (August 2014–January 2015)

## Surveillance of Carbapenem-*resistant Klebsiella pneumoniae*

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**Abstract**

In this study we report the analysis of 131 *Klebsiella pneumoniae* (*K. pneumoniae*) clinical isolates from patients hospitalized in various wards, of Perugia General Hospital, from August 2014 to January 2015. Forty two isolates (32.1 %), were resistant to at least one carbapenem antibiotic and, among these isolates, 14 (33.3 %) exhibited resistance to colistin. All isolates were carbapenemases producers and 41 (97.6 %) harboured the $bla_{KPC}$ gene. Carbapenem-resistant *K. pneumoniae* isolates (CRKPs) were, also, typed for the genotypic diversity and the results revealed the circulation of two major clusters.

This surveillance study evidences the spread of CRKP isolates in Perugia General Hospital and confirms that carbapenem-resistant *K. pneumoniae* isolates have reached epidemic dissemination in Italy. In addition the percentage of resistance to colistin resulted to be less than that observed in other hospital laboratories across Italy. In conclusion the circulation of these isolates should be monitored and appropriate policy of surveillance must be used, in a target manner, in order to reduce the spread of carbapenem-resistant isolates.

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**Keywords**
CRKP • KPC-KP • *K. pneumoniae* • Colistin • Carbapenemases

## 1 Introduction

Carbapenems (ertapenem, meropenem, imipenem, doripenem) are a class of β-lactam antibiotics with broad-spectrum antibacterial activity and are widely regarded as the drugs of choice for the treatment of severe infections caused by extended-spectrum beta-lactamase (ESBL)-producing Gram-negative bacteria (Paterson and Bonomo 2005; Nordmann et al. 2011). In recent years, carbapenem-resistant *Enterobacteriaceae* (CRE) emerged as one of the most challenging group of antibiotic-resistant pathogens. These isolates usually exhibit multidrug resistance phenotypes that leave very few therapeutic options (Hirsch and Tam 2010; Falagas et al. 2011). They cause infections associated with high rates of morbidity and mortality and have the potential for rapid dissemination in healthcare settings (Tzouvelekis et al. 2012; Nordmann et al. 2011).

The first carbapenemase producer in *Enterobacteriaceae* was identified in 1993 (Naas and Nordmann 1994), but over the last 10 years a rapid increase of carbapenemase producing *Enterobacteriaceae* (CPE) worldwide has been documented (Canton et al. 2012). This is, in part, due to the propensity of *Enterobacteria* to acquire genetic material through horizontal gene transfer, to the ease of transmission of such microorganisms among people (in particular through contaminated hands) and to the transfer of patients coming from hospitals where there had been outbreaks/epidemics caused by CPE.

Although the carbapenemases have been identified in various species of *Enterobacteriaceae* (Bratu et al. 2007), *K. pneumoniae* represents a major public health concern (Canton et al. 2012). The clinically most important carbapenemases in *K. pneumoniae* are the serine-β-lactamases (Class A), KPC type (*Klebsiella pneumoniae* carbapenemase), and the metallo-β-lactamases (MBL), (Class B) represented mainly by the types IMP (imipenemase), VIM (Verona integron-encoded metallo-beta-lactamase) and NDM (New Delhi metallo-beta-lactamase). Both, of these classes of enzymes, have been implicated in the rapid dissemination of carbapenem-resistant *K. pneumoniae* isolates (CRKPs) (Patel and Bonomo 2011). *Klebsiella pneumoniae* carbapenemase-producing *Klebsiella pneumoniae* (KPC-KP) cause serious infections in debilitated and immunocompromised patients with prolonged hospital stays. The mortality rates, range from 24 % to as high as 70 %, depending on the study population (Tzouvelekis et al. 2012; Patel et al. 2008). The respiratory tract is the most common site of infection (Mouloudi et al. 2010), however urinary infection, catheter related infection, surgical infection commonly, also, occur (Sievert et al. 2013). Since their discovery, 16 years ago (Yigit et al. 2001), KPC-KP isolates (KPC-KPs) had spread globally and in Italy have been documented for the first time in late 2008, where the likely source was a medical trainee from Israel (Giani et al. 2009). KPC-producing *K. pneumoniae* has since undergone rapid and extensive dissemination, associated, also, with outbreak of hospital infection (Fontana et al. 2010; Ambretti et al. 2010; Agodi et al. 2011). To control the spread of these bacteria in Italy, the Ministry of Health issued, in February 2013, a circular letter (Ministero della Salute 2013) asking the Italian regions to report all cases of bloodstream infections due to carbapenemase-producing *K. pneumoniae* or *E. coli* and recommending control measures to limit the spread in healthcare setting.

Polymyxins, together with tigecycline and gentamicin, are among the few agents that retain activity against CRKP, and are key components of the antimicrobial regimens that are recommended for treatment of infections caused by these pathogens. Recently, a major concern is

the emergence of colistin-resistant CRKP isolates (Ah et al. 2014; Kontopoulou et al. 2010; Munoz-Price et al. 2013; Mezzatesta et al. 2011). In Italy, Monaco et al. (2014) reported that, 43 % of KPC-KPs are resistant to colistin, highlighting the importance of monitoring this resistance.

In light of the worldwide emergence of CPE and, in particular in Italy, of the KPC-KPs, the aim of this retrospective study was to monitor the spread of these isolates in Perugia General Hospital, from August 2014 to January 2015, to set up interventions able to control and prevent the spread of these threatening microorganisms.

## 2 Materials and Methods

### 2.1 Study Design

Between August 2014 and January 2015, a total of 131 consecutive non-replicate clinical isolates of *K. pneumoniae,* recovered from patients hospitalized in Medical wards, Surgical wards, Intensive Care Units and Hematology/Oncology wards, were collected at the Clinical Microbiology Laboratory, of the Perugia General Hospital, Italy. The isolates were extracted from several diagnostic samples: urine, respiratory tract, blood, wound exudates or other biological material. According to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (Wayne 2014) and of European Antimicrobial Resistance Surveillance Network (EARS-Net) (ECDC 2013), we included in this study only the first isolate per patient, irrespective of the body site from which the specimen was obtained or the antimicrobial susceptibility pattern. Isolates from surveillance or screening cultures were excluded from the study.

The analysis of the data was performed by using of WHONET software, version 5.6, a software program for the management of microbiology laboratory data, that is available, free of charge, from the World Health Organization (WHO 2015).

### 2.2 Bacterial Strain Identification and Susceptibility Testing

Bacterial identification was carried out by Matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF MS) mass spectrometry (Bruker Daltonics, Bremen, Germany), as described elsewhere (Mencacci et al. 2013). Antimicrobial susceptibility testing was performed by using the Phoenix Automated Microbiology System (Becton Dickinson Diagnostic Systems, Sparks, United States) and the Vitek-2 System (bioMérieux, Marcy l'Etoile, France). Confirmatory MIC testing for meropenem, imipenem and ertapenem, was performed by Etest (bioMérieux). Antimicrobial MICs were interpreted using clinical breakpoints according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST 2015).

For carbapenem-resistant *K. pneumoniae* isolates MICs of colistin, tigecycline, gentamicin, and trimethoprim-sulfamethoxazole were determined by the broth microdilution SENSITITRE method (Oxoid, Hampshire, United Kingdom), according to the manufacturer's instructions. Antimicrobial MICs were interpreted according to the ongoing European Committee of Antimicrobial Testing (EUCAST) clinical breakpoints (EUCAST 2015).

### 2.3 Detection of Carbapenemases

All isolates with meropenem MIC >0.125 μg/ml was screened for carbapenemases production, according to EUCAST guidelines (EUCAST 2013). For phenotypic detection of carbapenemases production, the commercial KPC + MBL Confirm ID kit was used (Rosco Diagnostica A/S, Taastrup, Denmark) (Giske et al. 2011), employing: meropenem (MEM 10 μg), MEM + phenyl-boronic acid (PBA 600 μg), MEM + dipicolinic acid (DPA

1000 μg), and MEM + cloxacillin (CLOX 750 μg) disks. Briefly, a, 0.5 McFarland, inoculum was prepared from *K. pneumoniae* isolates spread on cation-adjusted Mueller–Hinton II agar plates (Becton-Dickinson) and the disks were placed on each plate. Results were interpreted according to the manufacturer's instruction. Particularly, production of KPC was considered when the growth inhibitory zone diameters seen around the meropenem disk with PBA had increased to ≥5 mm as compared to the growth inhibitory zone diameter seen around the disk containing meropenem alone. Production of metallo-β-lactamases (MBL) was considered when the growth inhibitory zone diameters seen around the meropenem disk with DPA had increased to ≥5 mm as compared to the growth inhibitory zone diameter seen around the disk containing meropenem alone.

Detection of $bla_{KPC}$ gene was performed by a commercial multiplex PCR (hyplex SuperBug ID) according to the manufacturer's directions (Amplex Diagnostics GmbH, Germany) (Kaase et al. 2012).

### 2.4 Isolate Genotyping

The isolates were typed using DiversiLab rep-PCR (bioMérieux, Marcy-l'Etoile, France), according to the manufacturer's instructions (Higgins et al. 2012a, b; Healy et al. 2005) and as previously described (Mencacci et al. 2013). Briefly, DNA was extracted from a bacterial culture by using the UltraClean Microbial DNA isolation kit, amplified using rep-PCR, loaded in LabChip and run using the Agilent 2100 Bioanalyzer (Agilent Technologies). The results were analyzed by DiversiLab system with the Pearson correlation (PC) coefficient to emphasize peak intensities more than peak presence or absence. The isolates were considered different, similar and indistinguishable, if the percentage of similarity was <95, 95–97.5 and >97.5 respectively.

## 3 Results

### 3.1 Carbapenem-Resistant *K. pneumoniae* Isolates: Distribution and Proportion in Hospital Wards

During the study period (August 2014–January 2015) a total of 131 consecutive non-replicate clinical isolates of *K. pneumoniae* were identified from patients hospitalized in the Perugia General Hospital. Among these isolates, 42 (32.1 %) were resistant to at least one carbapenem antibiotic (meropenem, imipenem or ertapenem). Total patients' mean age was 67 years (range: 1 month–95 years), 80 (61.1 %) males and 51 (38.9 %) females. Mean age of patients with CRKPs was 68 years (range: 2–93 years): 32 (76.2 %) males and 10 (23.8 %) females. Figure 1 shows the distribution of *K. pneumoniae,* total (panel a) or carbapenem-resistant (panel b) isolates, in the different wards of the Perugia General Hospital, the majority of them being from Medical wards.

The highest percentage of CRKPs, over the total number of *K. pneumoniae* isolated from the different wards, was observed in Hematology/Oncology (41.6 %; n = 5/12), followed by Medical wards (33.7 %; n = 26/77), Intensive Care Units (33.3 %; n = 5/15), and Surgical wards (22.2 %; n = 6/27).

### 3.2 Carbapenem-Resistant *K. pneumoniae*: Distribution and Proportion in Clinical Samples

Distribution of *K. pneumoniae* total isolates and CRKPs from biological source was evaluated. Among 131 total *K. pneumoniae* isolates, 53 were from urine, 28 from wound exudates, 25 from respiratory tract, 14 from blood, and 11 from other body sites. Distribution of the 42 CRKP isolates according to diagnostic samples and hospital wards is showed in Table 1. In Medical wards the highest number of CRKPs was obtained from

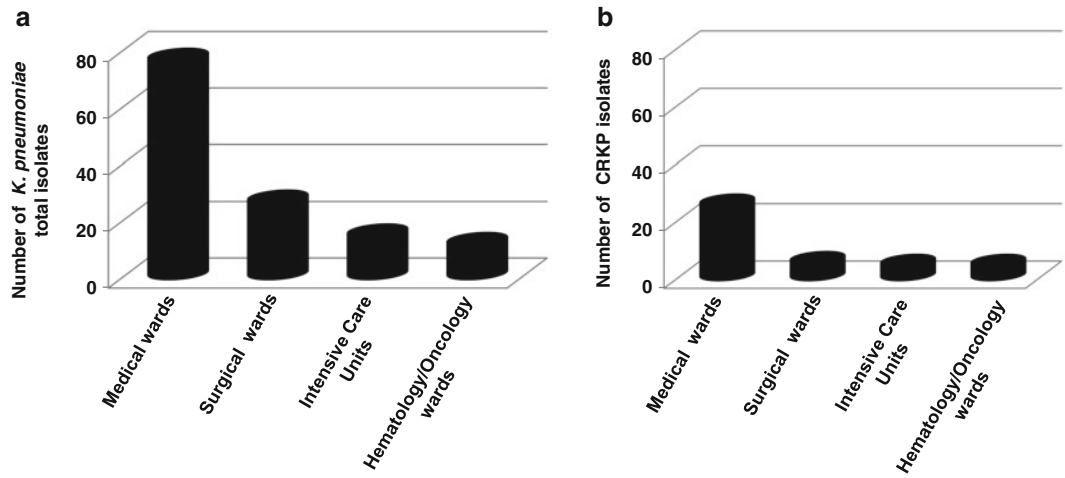


**Fig. 1** Distribution of *K. pneumoniae* total isolates (**a**) and carbapenem-resistant *K. pneumoniae* isolates (**b**) according to hospital wards

**Table 1** Distribution of 42 CRKP isolates according to diagnostic samples and Perugia General Hospital wards

| Wards | Number of CRKP isolates | | | | | |
|---|---|---|---|---|---|---|
| | Urine | Respiratory tract | Wound exudates | Blood | Others | Total |
| Medical wards | 13 | 7 | 2 | 3 | 1 | 26 |
| Surgical wards | 0 | 1 | 3 | 1 | 1 | 6 |
| Intensive Care Units | 1 | 3 | 1 | 0 | 0 | 5 |
| Hematology/Oncology wards | 0 | 0 | 2 | 3 | 0 | 5 |
| Total | 14 | 11 | 8 | 7 | 2 | 42 |

urine, all from urinary catheters, in Surgical wards from wound exudates, in ICU from respiratory tract, and in Hematology/Oncology wards from blood. The higher percentage of CRKPs, over the total *K. pneumoniae* isolates from the different diagnostic specimens, was observed for blood samples (50 %; n = 7/14), followed by respiratory tract (44 %; n = 11/25), wound exudates (28.6 %, n = 8/28), urine (26.4 %; n = 14/53), and other body sites (18.2 %; n = 2/11). The outcome of the patients with bacteremia was analysed and the results showed a mortality rate of 57.1 %.

## 3.3 Carbapenem-Resistant *K. pneumoniae* Isolates: Antimicrobial Susceptibility

Antimicrobial resistance profiles of the 42 - carbapenem-resistant *K. pneumoniae* isolates, according to EUCAST (EUCAST 2015) and CLSI (Wayne 2015) clinical breakpoints, is described in Table 2. Evaluation of MIC values for meropenem showed that, among these isolates, 38 exhibited a MIC >32 μg/mL, 2 showed MIC values of 16 μg/mL and 2 MIC values of 8 μg/mL, regarding imipenem 39 isolates exhibited MIC values >32 μg/mL and 3 MIC values of 8 μg/mL and regarding ertapenem all 42 isolates showed MIC values >32 μg/mL.

Antimicrobial susceptibility tests, against non-beta-lactam agents, revealed that 14 CRKP isolates were resistant to colistin (33.3 % versus 3.3 % exhibited by carbapenem-susceptible *K. pneumoniae* isolates), 9 exhibited resistance to gentamicin (21.4 %), 8 CRKPs were resistant to tigecycline (19 %), and 38 (90.5 %) were resistant to trimethoprim-sulfamethoxazole (Table 2). The colistin MICs were determined and the results showed that 20 isolates exhibited MIC values of 0.5 μg/mL, 8 MIC values of

**Table 2** Antimicrobial profile of carbapenem-resistant *K. pneumoniae* isolates (n = 42)

| Antimicrobial agents | Category | EUCAST Breakpoints[a] MIC (μg/mL) | EUCAST Number of isolates | CLSI Breakpoints[b] MIC (μg/mL) | CLSI Number of isolates |
|---|---|---|---|---|---|
| Meropenem | S | ≤2 | 0 | ≤1 | 0 |
| | I | 4–8 | 2 | 2 | 0 |
| | R | >8 | 40 | ≥4 | 42 |
| Imipenem | S | ≤2 | 0 | ≤1 | 0 |
| | I | 4–8 | 3 | 2 | 0 |
| | R | >8 | 39 | ≥4 | 42 |
| Ertapenem | S | ≤0.5 | 0 | ≤0.5 | 0 |
| | I | 1 | 0 | 1 | 0 |
| | R | >1 | 42 | ≥2 | 42 |
| Colistin | S | ≤2 | 28 | NA | NA |
| | I | NA | NA | NA | NA |
| | R | >2 | 14 | NA | NA |
| Gentamicin | S | ≤2 | 19 | ≤4 | 33 |
| | I | 4 | 14 | 8 | 4 |
| | R | >4 | 9 | ≥16 | 5 |
| Tigecycline | S | ≤1 | 12 | NA | NA |
| | I | 2 | 22 | NA | NA |
| | R | >2 | 8 | NA | NA |
| Trimethoprim/ Sulfamethoxazole | S | ≤2 | 4 | ≤2/38 | 4 |
| | I | 4 | – | – | – |
| | R | >4 | 38 | ≥4/76 | 38 |

*MIC* minimum inhibitory concentration, *S* susceptible, *I* intermediate, *R* resistant, *NA* not applicable
[a]MICs interpreted using clinical breakpoints according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2015
[b]MICs interpreted using clinical breakpoints according to Clinical and Laboratory Standards Institute (CLSI), 2015

1 μg/mL, 2 MIC values of 1 μg/mL and 12 isolates MIC values >16 μg/mL. The analysis of co-resistances against non-beta-lactam agents revealed that one isolate (2.3 %) was resistant to all four drugs, five isolates (11.6 %) were resistant to three drugs (two isolates to colistin, gentamicin and trimethoprim-sulfamethoxazole, two isolates to gentamicin, trimethoprim-sulfamethoxazole and tigecycline, one isolate to colistin, trimethoprim-sulfamethoxazole and tigecycline), thirteen isolates (30.2 %) were resistant to two drugs (six isolates to colistin and trimethoprim-sulfamethoxazole, three isolates to gentamicin and trimethoprim-sulfamethoxazole, three isolates to trimethoprim-sulfamethoxazole and tigecycline).

### 3.4 Phenotypic and Molecular Characterization of CRKP Isolates

All the CRKPs produced carbapenemase by phenotypic analysis: 41/42 isolates produced KPC type enzymes and one MBL type enzymes. All these isolates were analysed to determine the presence of $bla_{KPC}$ gene. The results showed that all but one (97.6 %) harboured the $bla_{KPC}$ gene. The genetic relatedness of these isolates, evaluated by rep-PCR using DiversiLab system, revealed five small clusters (Fig. 2, panel a): Cluster II and Cluster V, both with six isolates, Cluster I, III and IV each constituted by three isolates. The other isolates were not clonally

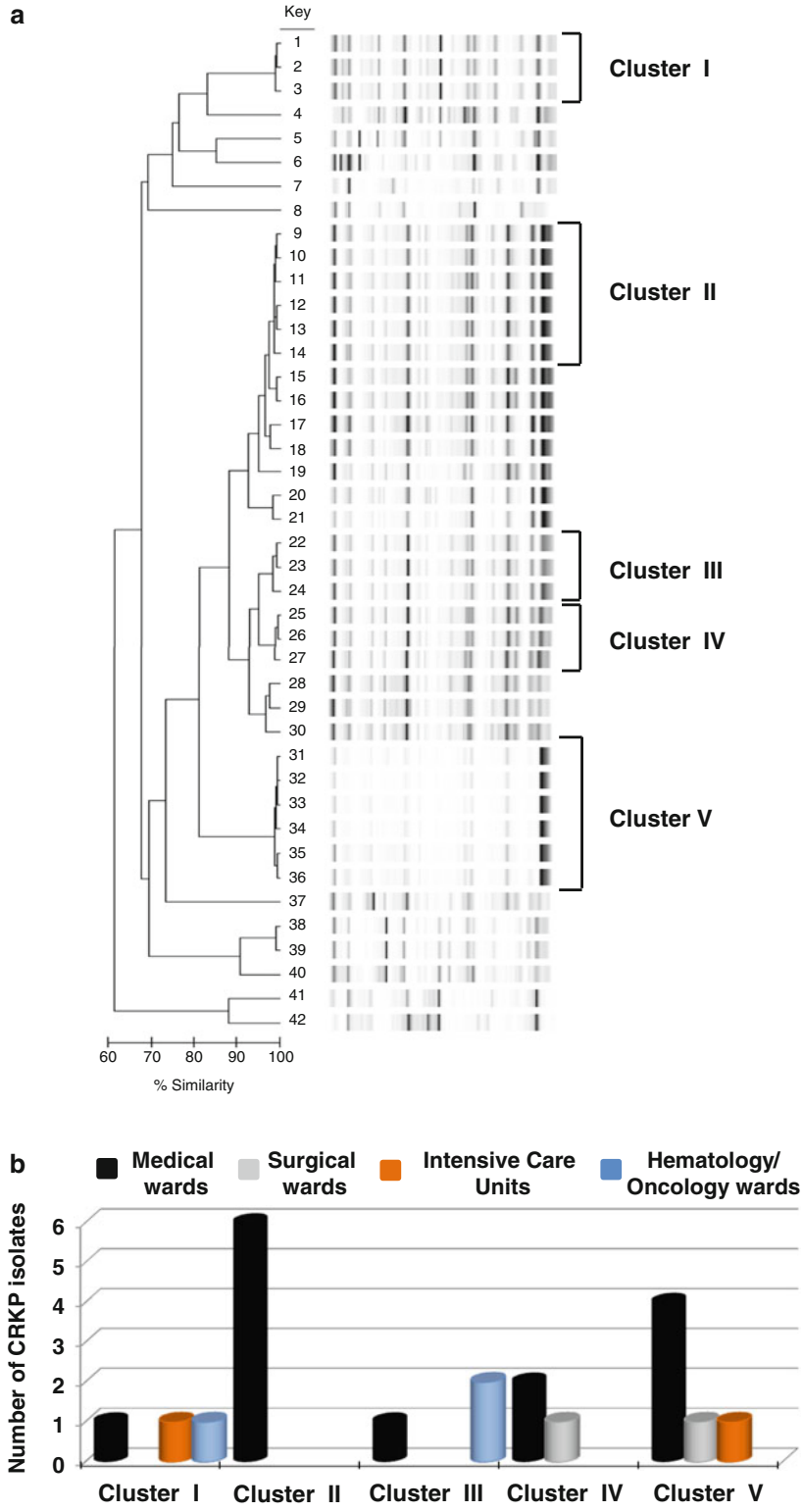


**Fig. 2** Genetic typing of carbapenem-resistant *K. pneumoniae* isolates (**a**) and distribution of relative clusters according to hospital wards (**b**)

related. Interestingly the isolates belonging to Cluster II were present in only the Medical Wards, while the isolates belonging to the other clusters were dispersed among the different hospital wards (Fig. 2, panel b). All isolates clustered harboured the $bla_{KPC}$ gene.

## 4 Discussion

The global dissemination of carbapenem-resistant *Enterobacteriaceae* is mostly due to isolates of *K. pneumoniae*. To date, CRKP isolates constitute a serious public health hazard and they are associated with a significant morbidity and mortality (Zarkotou et al. 2011).

In this study we report the analysis of 131 *K. pneumoniae* isolates recovered, from August 2014 to January 2015, in clinical specimens collected from patients hospitalized in various wards of Perugia General Hospital. Forty two isolates were CRKP (32.1 %), and, among these isolates, 14 (33.3 %) exhibited resistance to colistin. Carbapenemases production was detected in all CRKPs and 41/42 isolates (97.6 %) harboured the $bla_{KPC}$ gene. The analysis of CRKPs distribution, in different wards, showed that the dissemination of carbapenem-resistant *K. pneumoniae* isolates was not restricted to a high risk wards, but affected all major Perugia General Hospital sectors. Therefore the propensity to dissemination on multiple wards should be considered when planning infection control strategies.

The highest number of CRKPs was isolated from urine, followed by respiratory tract, wound exudates, blood and others specimens. Noteworthy, the higher percentage of CRKPs, over the total *K. pneumoniae* isolates, was observed for blood samples (50 %; n = 7/14). Moreover the outcome of the patients with bacteremia resulted in a mortality rate of 57.1 %. This suggests that an high percentage of *K. pneumoniae*, isolated from blood, have a remarkable potential, for causing life threatening infections. Various studies have focused the impact of bacteremia on the patient's outcome (Zarkotou et al. 2011; Giani et al. 2013). Tumbarello et al. (2012), described 125 patients with bloodstream infections due to KPC-positive isolates and reported a crude 30-day mortality of 42 %. Similarly, a study by Zarkotou et al. (Zarkotou et al. 2011), conducted in a Greece's hospital, revealed 53 bloodstream infections caused by KPC-positive *K. pneumoniae*, with a crude 30-day mortality of 53 % and an infection-attributable mortality of 34 %. In the light of these facts, to control the spread of KPC-KPs in Italy, the Ministry of Health issued a circular letter asking the Italian regions to report all cases of bloodstream infection due to *K. pneumoniae* and *E. coli*. To limit the spread in our hospital, an active screening of selected patient groups, including patients hospitalized in Hematology/Oncology wards and patients admitted to ICU, was performed. The infected/colonised patients, were isolated, from other patients and implementation of contact precautions, according to the recommendations issued at national and international level was observed (Monaco et al. 2014).

The analysis of distribution of the 42 CRKPs, isolated from different samples and hospital wards, showed that CRKPs recovered in Medical wards, were mostly from urine, those recovered in ICU were mostly from respiratory tract and those isolated in Hematology/Oncology wards were mostly from blood. This could be related to different devices used during the hospitalisation: urinary catheters in Medical wards, many other devices (intubation tubes, intravascular devices) in ICU and Hematology/Oncology wards.

As of today, few safe and practical therapeutic options remain for patients infected with KPC producers. Many clinicians have resorted to the use of tigecycline, polymyxins, and few remaining aminoglycosides (Kelesidis et al. 2008). A major concern is the emergence of colistin-resistant KPC-positive *K. pneumoniae* isolates. The situation is especially worrying since colistin is the core component of treatment combinations (Munoz-Price et al. 2013).

In Italy, the emergence of colistin-resistant KPC-KPs has been reported since 2010 and, in the first Italian nationwide cross-sectional survey

on CRE, carried out in mid-2011, the overall percentage of colistin-resistant, among KPC-KPs, was found to be 22.4 % (Giani et al. 2013). Recently, Monaco et al. (2014), in a surveillance study, reported that the percentage of colistin-resistant KPC-KP isolates, reached to 43 %. In this study we demonstrate that 33.3 % of CRKP isolates were resistant to colistin in respect to 3.3 % of carbapenem-susceptible *K. pneumoniae* isolates. In addition 19 % of CRKP isolates were resistant to tigecycline, 21.4 % were resistant to gentamicin and 90.5 % were resistant to trimethoprim-sulfamethoxazole. Our results evidence differences from previous reported data: in particular the percentage of colistin resistant isolates resulted lesser than that observed by Monaco et al. (2014). This suggests that the spread of colistin-resistant CRKPs, among Italian Hospital, may be different and support the importance of monitoring the trend of antimicrobial resistance of these isolates. Indeed, in a previous report, it has been highlighted the possibility of cross-transmission of colistin-resistant isolates even in the absence of colistin treatment and the lack of a substantial impact of colistin resistance on the bacterial fitness; as a consequence, clinically selected colistin-resistant organisms, once emerged, retain the potential to persist in the patients and in the hospital environment causing subsequent transmission (Bogdanovich et al. 2011).

It is well known that the understanding of geographical distribution and epidemiological typing tools of potential highly pathogenic isolates, are essential to rapidly locate the source and to trace the spread of hospital-associated pathogens. In this study the CRKP isolates were, also, typed using DiversiLab, a semi-automated rep-PCR-based system, that is, widely used to detect clonal relationships during a bacterial outbreak (Brolund et al. 2010; Corbellini et al. 2014; Ligozzi et al. 2010; Mencacci et al. 2013).

The genotypic analysis, performed in this study, revealed the circulation of two major clusters (II and V) and three minor clusters. Interestingly the isolates belonging to Cluster II were found only in Medical wards, suggesting that such transmission may have been related to medical devices used and/or care practices and/or procedures of cleaner and decontamination utilized in this type of wards. The isolates belonging to the other clusters were distributed through the hospital. The clusters were not related each other, indicating the absence of epidemiological relatedness between cases.

The results, of this surveillance study, show the spread of CRKP isolates in Perugia General Hospital and confirm that carbapenem-resistant *K. pneumoniae* isolates have reached epidemic dissemination in Italy. In addition the percentages of resistance observed, against last line drugs, in particular in respect of colistin, demonstrate that the circulation of these isolates must be monitoring to apply a policy of surveillance of antibiotic resistance in order to reduce the selective pressure that favors the emergence and the consequent spread of carbapenem-resistant isolates.

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# A Snapshot of Drug-Resistant *M. tuberculosis* Strains in Croatia

Ljiljana Zmak*, Mihaela Obrovac*, and Vera Katalinic-Jankovic

**Abstract**

Tuberculosis caused by resistant *M. tuberculosis* strains poses a serious threat as it requires prolonged and costly treatment and has high mortality rate. In order to investigate resistance to antituberculous drugs in Croatia, we analysed all resistant *M. tuberculosis* strains isolated from patients' samples in period 2010–2014 (1 strain per patient). Out of 2384 *M. tuberculosis* strains, we identified 88 (3.69 %) resistant strains. The analysis included resistance patterns, resistance conferring mutations and, according to MIRU-VNTR analysis, clustering and global lineages distribution. Relatively high number of strains was monoresistant, especially to isoniazid, while there were only six multiresistant strains. Among 59 strains with any pattern that includes resistance to isoniazid, a total of 22 (37.29 %) had resistance conferring mutation in *katG* gene (S315T), 23 (38.98 %) in *inhA* promoter region (C-15T) and 14 (23.73 %) had none of these mutations. The observed clustering rate of resistant strains was 28.41 %, and the most common global lineage was Euro-American (75 %).

**Keywords**

Resistant tuberculosis • Isoniazid resistance • Resistance conferring mutations • Clustering rate • Global lineages

Tuberculosis (TB) still represents a serious cause of morbidity and mortality worldwide. In the European Union and European Economic Area (EU/EEA), there were more than 40,000 reported TB cases in 2013, of which 10.7 % were caused by resistant *Mycobacterium* (*M.*) *tuberculosis* strains (ECDC 2015). TB caused by resistant *M. tuberculosis* strains (i.e. resistant TB) poses additional threat as it requires prolonged and costly treatment and has higher mortality rates then TB caused by sensitive strains.

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Approximately 500 new TB patients are identified in Croatia annually, resulting in incidence of 10 per 100,000 population in 2014 (CNIPH 2015). Although the percentage of resistant TB in Croatia is lower than the average in EU/EEA (3.5 % in 2013), these patients are closely monitored (ECDC 2015). For all bacteriologically confirmed patients, drug sensitivity testing (DST), as well as genotyping of isolated strains is routinely performed. Since 2007, all genotyping data is saved in the National genotyping database at the National Reference Mycobacteria Laboratory (NRL) (Croatian National Institute of Public Health, Zagreb), that contains information on more than 5000 *M. tuberculosis* strains. Until now, there was no comprehensive overview of *M. tuberculosis* strains resistance patterns or resistance conferring mutations in different genetic lineages in Croatia.

In order to investigate resistance to antituberculous drugs more in detail, in this study we included all resistant *M. tuberculosis* strains isolated from TB patients' samples in period 2010–2014 (1 strain per TB patient). No ethics committee approval was needed, as all the data resulted from the routine laboratory work of the NRL. Out of 2384 *M. tuberculosis* strains, in the study we included 88 (3.69 %) resistant strains. DST for first-line antituberculous drugs was performed using the proportion method on Lowenstein – Jensen solid medium according to Canetti, at the following final drug concentrations: isoniazid (INH) at 0.2 μg/ml, rifampicin (RMP) at 40 μg/ml, streptomycin (STM) at 4 μg/ml, ethambutol (EMB) at 2 μg/ml, and pyrazinamide (PZA) at 100 μg/ml (Canetti et al. 1969). RMP resistance conferring mutations in *rpoB* gene were determined using GenoType MTBDR*plus* assay (Hain, Germany). INH resistance conferring mutations were determined using *in house* multiplex PCR assay that detects *katG* S315T mutation and *inhA* promoter (C-15T) substitution (Herrera-León et al. 2005). All *M. tuberculosis* strains were genotyped by determining the copy number of 15-loci mycobacterial interspersed repetitive units – variable number of tandem repeats (MIRU-VNTR), according to the previously described protocol (Supply et al. 2006). *M. tuberculosis* global lineages of 88 resistant *M. tuberculosis* strains were identified by comparing their genotypes with reference strains in the MIRU-VNTR plus online tool (distance cut-off of <0.3, i.e. a difference in 7 loci) (Allix-Béguec et al. 2008; Weniger et al. 2010). At the same time, genetic tree of resistant *M. tuberculosis* isolates was constructed using the categorical index and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm (Fig. 1). *M. caprae* isolate from National genotyping database was included as outlier strain.

Out of 88 resistant TB strains, a total of 71 strains were monoresistant (80.68 %); 43 to INH, 27 to STM and one to RMP (30.68 %, 48.86 % and 1.14 %, respectively) (Table 1). Multiresistant pattern was identified in six (6.82 %) cases. In 10 (11.36 %) cases there was INH and STM resistance and in one (1.14 %) case there was resistance to STM and EMB. Among 59 strains with any pattern that includes resistance to INH, a total of 22 (37.29 %) had resistance conferring mutation in *katG* gene (S315T), 23 (38.98 %) in *inhA* promoter region (C-15T) and 14 (23.73 %) had none of these mutations (Table 2). The C-15T substitution in *inhA* promoter region was detected only in INH monoresistant strains, whereas mutation in *katG* gene and no mutation in these genes where detected in all INH resistance patterns. Moreover, we found that *katG* gene mutation was strongly associated with polyresistance, as nine out of ten strains resistant to INH and STM had that mutation. This finding is in concordance with results from van Soolingen et al. (2000), which showed that strains with mutations in *katG* are more likely to gain additional resistance (van Soolingen et al. 2000). In multiresistant strains, the most prevalent RMP resistance conferring mutation was S531L (in five cases), and in one case there was a mutation in codones 526–529 (Table 2).

Based on the data obtained from MIRU-VNTRplus online tool, 66 strains (75 %) belonged to the Euro-American global lineage (Table 2). The most prevalent sub-lineages were

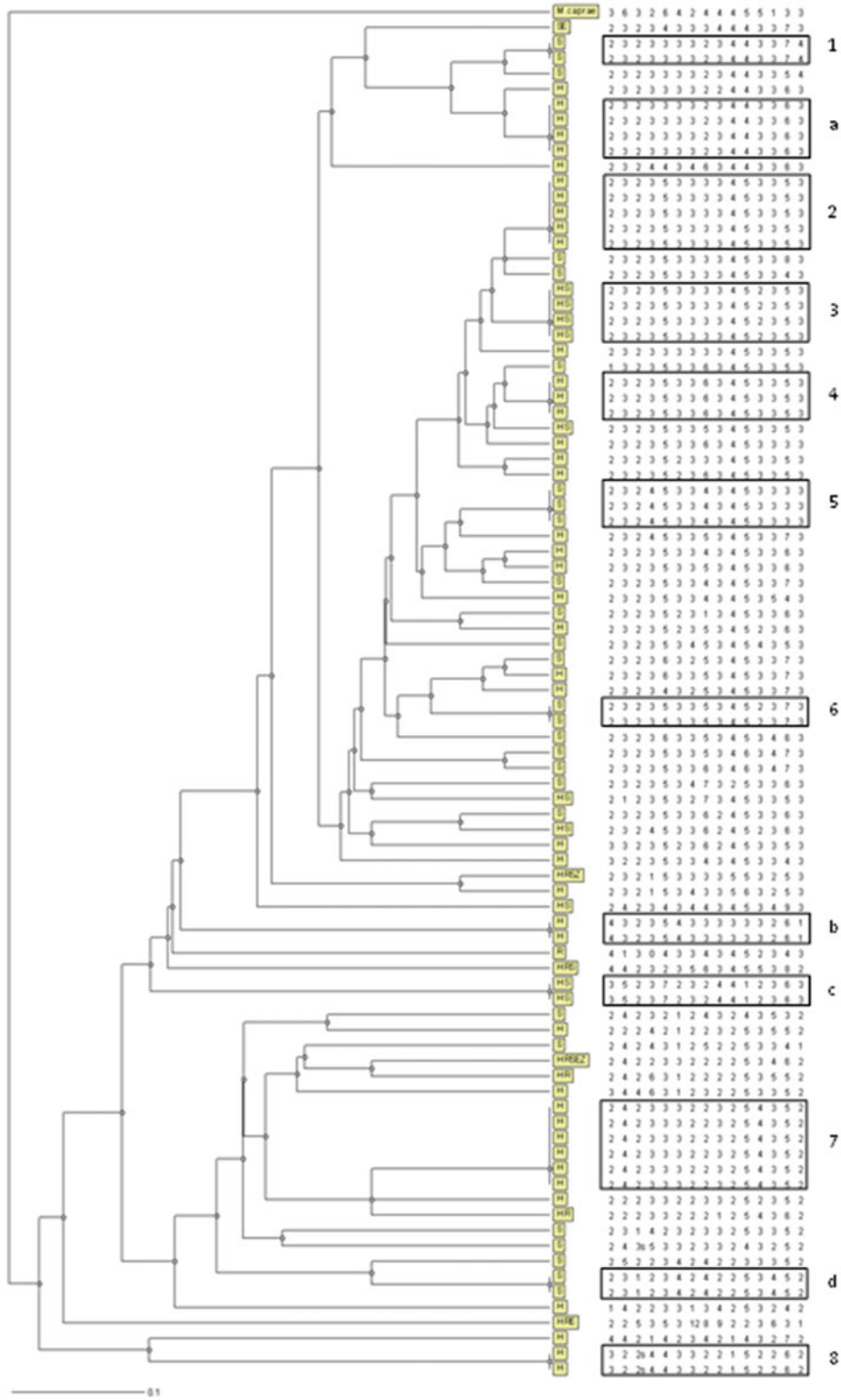


**Fig. 1** Genetic tree of 88 resistant *M. tuberculosis* isolates Allele numbers for each locus are in following order: 424, 577, 580, 802, 960, 1644, 1955, 2163b, 2165, 2401, 2996, 3192, 3690, 4052, 4156

**Table 1** Resistance patterns of *M. tuberculosis* strains in Croatia 2010–2014

| Resistance pattern | | % |
|---|---|---|
| **Monoresistance** | **71** | **80.68** |
| INH | 43 | 48.86 |
| STM | 27 | 30.68 |
| RMP | 1 | 1.14 |
| **INH+RMP multiresistance** | **6** | **6.82** |
| INH+RMP | 2 | 2.27 |
| INH+RMP+STM | 1 | 1.14 |
| INH+RMP+EMB | 1 | 1.14 |
| INH+RMP+STM+PZA | 1 | 1.14 |
| INH+RMP+STM+EMB+PZA | 1 | 1.14 |
| **INH+STM** | **10** | **11.36** |
| **STM+EMB** | **1** | **1.14** |
| **Patients total** | **88** | |

Haarlem (53 strains, 60.22 %), followed by Uganda I&II (7 strains, 7.95 %) and Latin American – Mediterranean (3 strains, 3.41 %). Two (2.27 %) strains belonged to URAL sub-lineage, while one (1.14 %) strain belonged to Ghana and one (1.14 %) to X sub-lineage. East-African-Indian and Beijing sub-lineages were detected in two cases (1.14 % each), for two (2.27 %) strains there were multiple matches and for 17 (19.32 %) strains the global lineage was not determined. These findings were expected, as in our previous study, where we analyzed genetic lineages of 1587 *M. tuberculosis* strains, the most prevalent global lineage was Euro-American, and the most

**Table 2** Genetic lineages, resistance patterns and INH and RMP resistance conferring mutations of *M. tuberculosis* strains in Croatia 2010–2014

| Global lineage | Sub-lineage | No. (%) of isolates | Resistance pattern | INH resistance conferring mutation | RMP resistance conferring mutation |
|---|---|---|---|---|---|
| **1. Indo-Oceanic** | **East-African-Indian** | **1 (1.14)** | 1 INH+RMP +EMB | WT | S531L |
| **2. East-Asian** | **Beijing** | **1 (1.14)** | 1 INH+RMP +STM | WT | Δ526-529 |
| **3. Euro-American** | **Lineage 4 total** | **66 (75.00)** | | | |
| | **Haarlem** | 53 (60.22) | 26 INH | 16 *inhA* C-15T, 6 *katG* S315T, 4 WT | – |
| | | | 18 STM | – | – |
| | | | 7 INH+STM | 6 *katG* S315T, 1 WT | – |
| | | | 1 STM+EMB | – | – |
| | | | 1 INH+RMP +STM+EMB +PZA | *katG* S315T | S531L |
| | **Latin American – Mediterranean** | 3 (3.41) | 3 INH | 3 *katG* S315T | – |
| | **Uganda I&II** | 7 (7.95) | 7 INH | 7 *inhA* C-15T | – |
| | **URAL** | 2 (2.27) | 2 INH+STM | 2 *katG* S315T | – |
| | **Ghana** | 1 (1.14) | INH | WT | – |
| | **X** | 1 (1.14) | INH+STM | *katG* S315T | – |
| **4. Other** | **Multiple matches** | **2 (2.27)** | 2 STM | – | – |
| | **Unknown** | **17 (19.32)** | 7 STM | – | – |
| | | | 6 INH | 2 *katG* S315T, 4 WT | – |
| | | | 1 RMP | – | WT |
| | | | 2 INH+RMP | 2 WT | 2 S531L |
| | | | 1 INH+RMP +STM+EMB +PZA | *katG* S315T | S531L |
| **Total** | | **88 (100)** | | | |

prevalent sub-lineage Haarlem (Zmak et al. 2014). Regarding the relation of resistance patterns and genetic lineages, the most heterogeneous sub-lineage was Haarlem, according to the presence of various resistance patterns (Table 2). Other sub-lineages were not as heterogeneous, as all strains belonging to e.g. Uganda I&II sub-lineage not only were monoresistant to INH, but had the same INH resistance conferring mutation (Table 2). Surprisingly, although the majority of resistant strains belong to the Euro-American global lineage, only one out of six multiresistant strains was found to belong to Haarlem sub-lineage. Both East-African-Indian and Beijing strains were multiresistant and isolated in foreign citizens residing in Croatia. Based on epidemiological data, it can be presumed that these strains were imported to our country. To date, there were no new TB cases reported caused by strains of these genotypes.

To determine the clustering rate of resistant *M. tuberculosis* strains, all 88 genotypes were analyzed in comparison to the National genotyping database. A total of 13 strains were clustered only with resistant strains; three of them were clustered in three different clusters with resistant strains that were isolated before 2010. Out of remaining 10 strains, there were three clusters of two strains each, and one cluster of four resistant strains (Fig. 1, clusters a–e). Another 49 strains were clustered with sensitive strains and among them, 22 were the only resistant strains in 22 different clusters. These strains were not included in calculation of clustering rate, because we assume there was no transmission of resistant strain. Remaining 27 strains were part of eight different clusters: two clusters with two resistant strains each, two clusters with three resistant strains, and three clusters with four, five and six resistant strains clustered with sensitive strains, respectively (Fig. 1, clusters 1–8). The proportion of clustered resistant isolates was calculated using the equation: (number of clustered strains – number of clusters)/total number of strains, giving the clustering rate of 28.41 %. A total of 26 (29.54 %) resistant strains were of unique genotype. The clustering rate is slightly higher than the one found in our previous study (18.2 %) where a smaller number of resistant strains (n = 64) were analyzed (Zmak et al. 2014). In the present study, longer period of time was observed and the higher clustering rate of resistant strains probably mirrors more accurately the resistant TB transmission in Croatia.

This is the first report that gives a snapshot of resistance patterns, clustering and genetic lineages of resistant *M. tuberculosis* strains circulating in Croatia. Although we found a low number of patients with resistant TB, there was relatively high number of monoresistant strains identified, especially to INH. The importance of this finding is highlighted by the study from Espinal et al. (2000) that emphasized association between any INH resistance other than multidrug resistance and unfavorable treatment outcome. Moreover, these patients have to be monitored with caution, as there is the possibility of developing resistance to RMP and becoming multiresistant.

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# Quorum Quenching Strategy Targeting Gram-Positive Pathogenic Bacteria

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**Abstract**

Quorum sensing (QS) is a cell density-dependent regulatory system that orchestrates the group behavior of unicellular organisms by synchronizing the expression of certain gene(s) within the clonal community of same species. Bacterial pathogens often employ QS system to establish efficiently an infection. A large part of low GC Gram-positive bacteria belonging to phylum Firmicutes use thiolactone/lactone peptides as communication signals so-called autoinducing peptides (AIPs) to coordinate QS circuit. In particular, QS of staphylococci, enterococci, and clostridia have been intensively studied in terms of alternative target of anti-pathogenic chemotherapy independent of bactericidal antibiotics. Thus far, a number of quorum quenching (QQ) agents that targeting the QS circuit of these Gram-positive pathogens have been developed by random screening of natural compounds or rationale design of AIP antagonists. This review summarizes those QQ agents and previews their potential as post-antibiotic drugs.

**Keywords**

Quorum sensing (QS) • Quorum quenching (QQ) • Autoinducing peptide (AIP) • Gram-positive bacteria

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## 1 Introduction

To synchronize the function of each cell in a community, unicellular organisms employ a quorum sensing (QS) system in which diffusible, extracellular signals control the expression of certain gene(s) (Bassler 1999). Gram-positive bacteria often employ extracellular signals such as peptides to control QS, which are known as the "auto-inducing peptides (AIPs)" (Otto 2001). The chemical structure of AIPs is diversified into several types, such as small oligopeptides, cyclic lactone/thiolactone peptides, and bacteriocin-like peptides which includes lantibiotics (Bierbaum and Sahl 1993; Nakayama et al. 2001; Novick 2003; Ma et al. 2015). With the exception of small oligopeptides, which are internalized as an intact form into the cytosol and modulate gene expression either directly or indirectly, most AIP signals are transmitted via a two-component regulatory system consisting of a histidine kinase receptor that is integrated into the membrane and a cytosolic response regulator that controls the expression of target gene (s) (Grebe and Stock 1999; Cheung et al. 2009). Thiolactone/lactone AIPs are widely used by Firmicutes, which include some serious and/or problematic pathogens such as staphylococci, enterococci, clostridia, and listeria (Novick and Muir 1999; Nakayama et al. 2001; Autret et al. 2003; Ma et al. 2015). Some of these pathogens are developed antibiotic resistance, including methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant enterococci (VRE), and often cause hospital epidemics when they present as nosocomial infections (Eguchi et al. 2009). Several antibiotic-resistant strains of *Clostridium perfringens* have also been identified and are abundantly found in broilers (Gholamiandehkordi et al. 2009). These circumstances have motivated the development of new types of chemotherapies like quorum quenching (QQ), which can act partially or completely independent of antibiotics.

Thus far, a large number of studies have been performed to develop QQ compounds targeting the cyclic peptide-mediated QS. By screening from prokaryotes or eukaryotic bioresources, various kinds of bioactive compounds have been identified as QQ agents (Nakayama et al. 2007, 2009; Mansson et al. 2011; Gordon et al. 2013; Desouky et al. 2013, 2015). These showed various mode of actions, each of them blocking different site of QS system (Table 1). Rationale design of QQ molecules were also successful, in particular, AIP antagonists have been developed for all of *S. aureus*, *E. faecalis* and *C. perfringens* (Lyon et al. 2000; Nakayama et al. 2013; Tal-Gan et al. 2013b, 2014; Singh et al. 2015). Some developed QQ compounds are efficiently quenched the QS at practical dose range such as nanomolar or sub-nanomolar concentrations and were demonstrated to be effective *in vivo* as well as *in vitro* (Balaban et al. 2003; Tal-Gan et al. 2013b, 2014; Nakayama et al. 2013).

In this review, we show examples and future perspective of QQ agents targeting the cyclic peptide-mediated QS of these low GC Gram-positive pathogens.

## 2 QQ Strategies Targeting the *agr*-like QS System of Gram-Positive Bacteria

Among a number of the cyclic peptide mediated QS system of Gram-positive bacteria, *agr* system of *S. aureus* is best characterized as shown in Fig. 1. Four genes involved in the QS system are encoded in an accessory gene regulator (*agr*) locus (Benito et al. 2000). The propeptide of AIP is translated from *agrD* and is then processed by a cell membrane enzyme encoded by *agrB*. Eventually, mature AIP with thiolactone bridge is secreted outside the cell. With the increase of the bacteria cells, the concentration of AIP increases, and when cell numbers reach at a certain threshold, AIP triggers activate two-component regulatory system encoded by *agrC* and *agrA*. The activated AgrA promotes the expression of RNAIII, which is a regulatory RNA molecule as well as

**Table 1** Some of the potential QQ AIP analogous or compounds have been reported so far against Gram-positive bacterial strains of *Staphylococcus aureus, Enterococcus faecalis* and *Clostridium perfringens*

| Known inhibitor | Strain tested and $IC_{50}$ for QQ | Activity | Reference |
|---|---|---|---|
| **AIP analogous against *Staphylococcus aureus*** | | | |
| AIP-II | Group- 1- RN6390B, (2.9 ± 1.2 nM) | Inhibiting β-lactamase; | Mayville et al. (1999) |
| | Group- II-SA502A, | In vivo: s.c. abscess was assayed in a mouse protection test. | |
| | Group- III-RN8463 (3.2 ± 1.3 nM) | | |
| AIP-II-N3A | Group- II-SA502A (180 nM) | Inhibiting -β-lactamase | Mayville et al. (1999), Lyon et al. (2000) |
| AIP-II truncate | RN9222(CA1-I) (272 ± 67 nM) | Inhibiting β-lactamase except RN8465, instead used for α- toxin production. | Lyon et al. (2000) |
| | RN9372(CA2-II) (209 ± 39 nM) | | |
| | RN8465 (Group III) (10 ± 1 nM) | | |
| | RN9371 (CA4-IV) (188 ± 50 nM) | | |
| AIP-1-D5A | Group- 1- RN6390B | Inhibition of production of toxic shock syndrome toxin (TSST-1) and enterotoxin C3 | McDowell et al. (2001) |
| Hapten-linked AIP-IV (AP4-24H11) | Group- I- Wood 46 | Reducing α-toxin production in both strains and severity of dermonecrosis in a skin and soft tissue infection mouse model of RN4850 | Park et al. (2007) |
| | Group- IV- RN4850 | | |
| AIP-III D4A | Group- I- AH1677 (0.485 nM) | Hemolysis and TSST-1 inhibition and used strains were- | Tal-Gan et al. (2013b) |
| | Group- II- AH430 (0.429 nM) | Group-I- RN6390B7 | |
| | Group- III- AH1747 (0.0506 nM) | Group-II- RN6923 | |
| | Group- IV- AH1872 (0.0349 nM) | Group-III MN8 | |
| | | Group-IV RN4850 | |
| tAIP-I D2A | Group- I- AH1677 (3.06 nM) | Hemolysis and TSST-1 inhibition and used strains were- | Tal-Gan et al. (2013b) |
| | Group- II- AH430 (10.1 nM) | Group-I- RN6390B7 | |
| | Group- III- AH1747 (0.260 nM) | Group-II- RN6923 | |
| | Group- IV- AH1872 (0.353 nM) | Group-III MN8 | |
| | | Group-IV RN4850 | |
| AIP-III nF5 | Group- I- AH1677 (13.8 nM) | β-lactamase inhibition and used strains were- | Tal-Gan et al. (2014) |
| | Group- II- AH430 (75.6 nM) | Group-I RN9222 | |
| | Group- III- AH1747 (>1000 nM) | Group-II RN9372 | |
| | Group- IV- AH1872 (0.839 nM) | Group-III RN9532 | |
| | | Group-IV RN9371 | |
| cyclo(L-Tyr-L-Pro) and cyclo (L-Phe-L-Pro) | *S. aureus* strain RN4220 | Inhibiting production of TSST-1 | Li et al. (2011) |
| **RNA III inhibiting peptide (RIP) against *S. aureus*** | | | |
| YSPWTNF | *S. aureus* 8325–4 | Reduced keratitis (tested in rabbits against 8325–4), osteomyelitis (tested in rabbits against MS), mastitis (tested in cows against Newbould 305, AE-1), and environmental infections and septic arthritis (tested in mice against LS-1) | Balaban et al. (2000a) |
| | *S. aureus* MS | | |
| | *S. aureus* Newbould 305, AE-1 | | |
| | *S. aureus* LS-1 | | |

(continued)

**Table 1** (continued)

| Known inhibitor | Strain tested and $IC_{50}$ for QQ | Activity | Reference |
|---|---|---|---|
| YSPWTNF-amide | *S. aureus* RN6390B (ATCC 55620) | Inhibit RNAIII *in vitro* and cellulitis *in vivo* (tested in Balb/C mice) | Gov et al. (2001) |
| | *S. aureus* Smith Diffuse | | |
| | *S. aureus* RN6911 | | |
| | *S. aureus* RN833 (ATCC 55619) | | |
| Tetra-peptide, SPWT | *S. aureus* strain Smith diffuse | Subcutaneous rat pouch model of infection | Baldassarre et al. (2013) |
| First with RIP and then antibiotics | *S. aureus* strain Smith diffuse | Reduced biofilm upto 1 fold and bacteremia was not detected in rat model of central venous catheter (CVC) | Cirioni et al. (2006) |
| **AIP analogous against *Enterococcus faecalis*** | | | |
| ZBzl-YAA5911 | *E. faecalis* ORIRF (26.2 nM) | Aphakic rabbit endophthalmitis model | Nakayama et al. (2013) |
| [$Ala^{5,9,11}$]-Z-GBAP | *E. faecalis* ORIRF (8.7 μM) | – | |
| **AIP analogous against *Clostridium perfringens*** | | | |
| Z-$AIP_{Cp}$-L2A/T5A and Z-$AIP_{Cp}$-F4A/T5S | *C. perfringens* 13 (type-A) 0.32 and 0.72 μM respectively | Inhibition of *pfoA* transcription | Singh et al. (2015) |
| **Natural inhibitors** | | | |
| Siamycin and Ambuic acid | *E. faecalis* OU510 and OG1RF | Inhibition of gelatinase production | Nakayama et al. (2007), (2009) |
| Hamamelitannin | Methicillin-resistant *S. aureus* (MRSA) | Preventing device-associated infections *in vivo*; RIP analogous. | Kiran et al. (2008) |
| cyclo(L-Tyr-L-Pro) and cyclo (L-Phe-L-Pro) | *S. aureus* strain RN4220 | Inhibition of production of TSST-1 | Li et al. (2011) |
| 3- tetra-decanoyltetronic | *S. aureus* | Reducing nasal cell colonization and arthritis in a murine infection model. | Murray et al. (2014) |
| | Group- 1- RN6390B | | |
| Avellanin C obtained from the fungus *Hamigera ingelheimensis* | *S. aureus agr* reporter strain (8325–4) with an $IC_{50}$ value of 4.4 μM | Reduction of *agr*-signaling pathway | Igarashi et al. (2015a) |
| Arthoamide from *Arthrobacter* Sp. | *S. aureus agr* reporter strain (8325–4) with an $IC_{50}$ value of 0.3 μM | Reduction of *agr*-signaling pathway | Igarashi et al. (2015b) |
| ω-hydroxy-emodin from *Penicillium restrictum* | Group- I- MRSA strain USA300 LAC | Reducing dermonecrosis and inflammatory cytokine transcription in mouse model of skin and soft tissue infection | Daly et al. (2015) |
| Cyclodepsipeptide (WS9326A) | *E. faecalis* OU510 and OG1RF (2.7 μM); *S. aureus* strains 8325–4 (type-I AIP), K12 (type-II AIP), and K9 (type-IV AIP) (19 μM) as well as *C. perfringens* 13 (type A) (0.88). | Inhibition of gelatinase production in *E. faecalis*; Reducing inhibition of hemolysis in *S. aureus*; inhibiting transcription of *pfoA* in *C. perfringens*. | Desouky et al. (2015) |
| Cyclodepsipeptide (WS9326B) | *S. aureus* strains- Newman (type-I) and K3 (type-II) | Attenuating the corneal cytotoxicity of *S. aureus* | Desouky et al. (2015) |

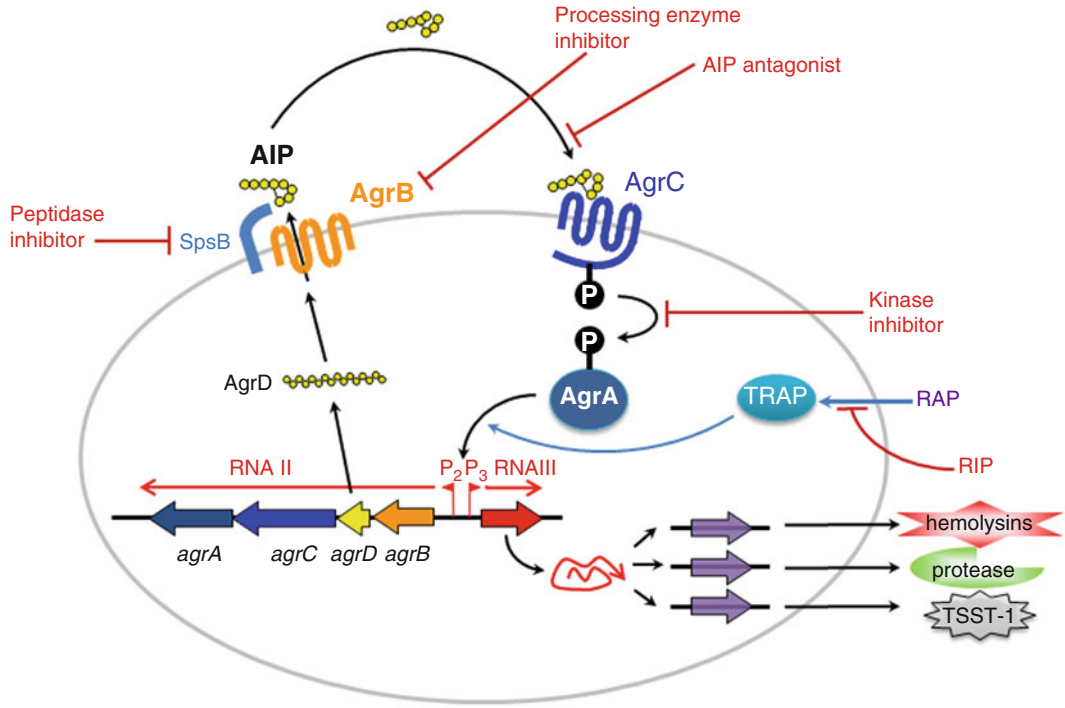


**Fig. 1** Molecular mechanism of the *agr* QS system and target of QQ agents in *Staphylococcus aureus*. The *agr* locus is composed of two divergent transcripts, called RNAII and RNAIII, which are driven by the P2 and P3 promoters, respectively. The RNAII transcript is an operon with four genes that encode for the core machinery of the QS system. Pro-AgrD (the autoinducer peptide, AIP) is processed and exported by AgrB. AgrB catalytically functions as a cysteine endopeptidase that processes pro-AIP into AIP and is secreted out of bacterial cells with the help of SpsB. At the threshold concentration, AIP binds to the AgrC receptor, which is a membrane-bound histidine kinase and activates the AgrC kinase. Thereafter, the histidine kinase domain of the AgrC kinase induces phosphorylation of the AgrA response regulator and subsequently activates the P2 and P3 promoters. RAP induces and RIP inhibits the phosphorylation of TRAP, thus, both may compete for binding to the same site on the receptor. Current strategies to attenuate virulence gene expression can be grouped into different categories: (1) inhibition of catalytic functions of AgrB and SpsB, (2) competitive inhibitors of AgrC via natural QSI agents or synthesized AIP analogs, (3) blocking of histidine kinase activity, (4) inhibition of AgrA – P2/P3 interactions, and (5) RNAIII transcriptional inhibitors

mRNA of δ-hemolysin. Eventually, RNAIII transcriptionally or translationally controls the expression of a series of genes involved in virulence (Recsei et al. 1986; Novick et al. 1993). It is further known that there are complicated cross-talk networks to the *agr* system. Among them, RNAIII-activating protein (RAP)-target of RAP (TRAP) system have been intensively studied as a target of anti-biofilm agent called RAP-inhibiting peptide (RIP) (Gov et al. 2001; Giacometti et al. 2005; Cirioni et al. 2006; Anguita-Alonso et al. 2007).

*Agr*-like gene cluster consisting of the four genes wholly or in part have been found in genomes of a series of low GC Gram-positive bacteria belonging to phylum Firmicutes (Kleerebezem et al. 1997; Shimizu et al. 2002; Lyon and Novick 2004). *Enterococcus faecalis* has the cognate gene locus named *fsr*, while an operon encoding two pathogenicity-related extracellular proteases. This operon is present immediately downstream of the *fsr* locus and its transcription is directly controlled by the FsrCA two-component regulatory system, not via a regulatory RNA like RNAIII in staphylococci (Nakayama et al. 2001, 2006). *Agr*-like gene cluster has been also found from a number of clostridial species, e.g., *C. perfringens*,

*C. difficile* and *C. botulinum* (Shimizu et al. 1994; Cooksley et al. 2010; Martin et al. 2013). In the case of *C. perfringens*, an operon of two component regulatory system termed VirSR is present independently from an *agrBD*-like gene cluster encoding AIP propeptide and its processing enzyme, while a series of virulence genes are controlled under the VirSR system associated to its downstream complicated network of regulatory RNAs (Ba-Thein et al. 1996; Ohtani et al. 2002, 2010; Ohtani and Shimizu 2014).

Figure 1 shows the possible target sites in the *agr* QS system for the development of QQ agents. Biosynthesis of AIP would be the first choice because it is the initial event in the QS signal circuit (see Sect. 5). Two enzymes, AgrB and SpsB are known to be involved in the biosynthesis of AIP (Zhang and Ji 2004; Kavanaugh et al. 2007). AgrB is specifically involved in the biosynthesis of cyclic AIP, while SpsB is type-I signal peptidase involved in the secretion of Sec- and Tat-dependent proteins (Auclair et al. 2012). These are processing enzymes with protease activity and inhibition of those catalytic functions is expected to block entire QS signaling. However, enzyme inhibitors appear to be more or less bactericidal or bacteriostatic even if targeting AgrB that is specific to AIP biosynthesis rather than SpsB that is common for wide-range of secretary protein. Blockage of AgrC-AgrA two component regulatory system would be the second choice (see Sect. 3.1). Receptor antagonists of AgrC are most probably developed based on analogue inhibitor as a template, (Novick and Muir 1999; Lyon et al. 2000, 2002; George and Muir 2007; Geisinger et al. 2008; Gordon et al. 2013; Tal-Gan et al. 2013b). These peptide antagonists are expected to have high specificity to AgrC and neither bactericidal nor bacteriostatic activity. Kinase inhibitors targeting AgrC are also possible but appear to have bactericidal and/or bacteriostatic activity because a number of different histidine kinases are functioning in bacteria. The above three targets are available for *agr*-like systems of other Gram-positive bacteria, while there are some targets specific to each bacterial group, such as AgrA – P2/P3 interaction in staphylococci (Sully et al. 2014) or TRAP in *S. aureus* (Balaban et al. 2001) (see Sect. 4). Some of the potential QQ AIP analogous or compounds have been mentioned in the table 1.

## 3 Development of AIP Antagonist Based on Structure-Activity Relationship Study

### 3.1 AIP Antagonists Targeting Staphylococcal *agr* System

Genome wide studies have revealed the presence and diversity of the *agr* gene clusters in staphylococci (Ji et al. 1997; Jarraud et al. 2000; McDowell et al. 2001). AIPs in the *agr* QS system commonly consist of macrocyclic and exocyclic tail moieties, which consist of five and two to four amino acid residues, respectively (Fig. 2). These AIPs control the expression of a number of virulence-related genes (Janzon and Arvidson 1990; Novick et al. 1993, 1995; George and Muir 2007). There are strain variations in the sequence of the *agr* gene cluster, especially in *agrD* and *agrC*, which encode for an AIP and a histidine kinase receptor, respectively (Ji et al. 1997; Cisar et al. 2009). Thus far, four AIP groups (AIP-I–IV) were identified based on the specificity between the AIP and AgrC, among which interference by non cognate AIP groups were observed (Ji et al. 1997). Notably, the discovery of the bacterial interference phenomenon between different *Staphylococcal* strains that secret AIPs with different structures have accelerated the rational design of AIP antagonists (Ji et al. 1997; Lyon et al. 2000; Tal-Gan et al. 2013b, 2014).

QS inhibitors that attenuate the virulence response in *S. aureus* were developed by (1) chimeras that were constructed by modifying natural AIPs using other amino acids and (2) removing the AIP tail that eventually inhibits AgrC activation as mentioned in the Fig. 2 (Mayville et al. 1999; Lyon et al. 2000; Gordon et al. 2013; Tal-Gan et al. 2013b, 2014).

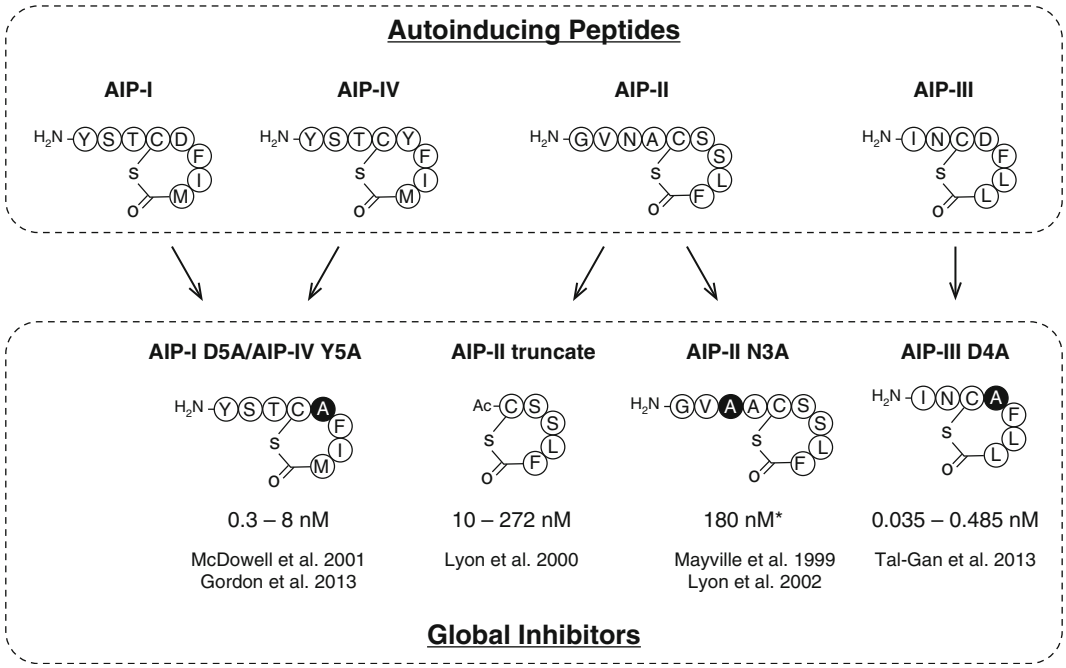


**Fig. 2** The structure of AIPs and the designed global inhibitors. Representative AIPs and designed global inhibitors are shown. *Black circles* represent amino acids that were replaced from one template AIP. Range of $IC_{50}$ values for all four AgrC groups are shown under the inhibitor structure

Mayville et al. (1999) began a structure-activity analysis on AIPs and inhibitors and found that amino acid residue replacement of the AIP-II tail region increased its inhibition activity. In particular, the AIP-II N3A region showed global inhibitor activity (Fig. 2). Lyon et al. (2000) found a truncated AIP-II that lacked the tail moiety and was shown to be a global AIP antagonist that inhibited the *agr* QS of all four AIP groups, suggesting that the ring region and tail region are important for receptor binding and receptor activation, respectively (Fig. 2). Namely, it can be understood that the macrocycle functions as the 'address' region and is required for molecule recognition, while the N-terminal exocyclic tail is the 'message' region and is necessary for AgrC activation. This hypothesis was supported by a series of studies that followed the structure-activity relationship (SAR) of AIPs (McDowell et al. 2001; Tal-Gan et al. 2013a, 2014) and their receptors (Wright et al. 2004; Jensen et al. 2008; Geisinger et al. 2008). AgrC is a membrane protein that has six membrane spanning regions and three extracellular loops (loop 1–3 from the N-terminus) and this topology is predicted to be conserved among AgrCs from all AIP groups (Lyon et al. 2000; Wright et al. 2004; Geisinger et al. 2008). Site-directed mutagenesis experiments indicate that loop 2 is involved in the specific recognition of AIP, while loop 1 is essential for receptor activation by the cognate AIP (Jensen et al. 2008; Geisinger et al. 2008). According to these data, we can speculate that the AIP ring region addresses the extracellular loop 2 of AgrC in order for the tail to trigger conformational changes in loop 1, which eventually results in the activation of the intracellular kinase domain through autophosphorylation. For instance, the interaction of the AgrC-1 and AgrC-4 receptors with their cognate AIP-I and AIP-IV or with non-cognate AIPs from *S. aureus* strains have shown that respective AIPs are activators of their cognate AgrC receptors but can also inhibit the non-cognate AgrC receptors at an $IC_{50}$ of 7–11 nM (Jensen et al. 2008).

McDowell et al. (2001) found that substitution of Asp-5 with alanine transformed AIP-I into a potent global antagonist with a nanomolar-level $IC_{50}$ against all four AgrC subtypes. AIP-1 and AIP-4 differ at the 5th position, which was suggested to be a determinant of AIP specificity for these two groups (Fig. 2). The 5th position can be substituted with bulky amino acids while maintaining its agonist activity, suggesting a model where the AgrC receptor is switched on when it recognizes sufficient steric bulk at the second endocyclic position in AIP-1. Johnson et al. (2015) targeted the Asp-5 in AIP-1 and substituted it with either β-alanine (β-ala) or L-β-homoalanine (hβ-ala). The β-ala substitution of Asp-5 completely lost both its agonist and antagonist activities. Interestingly, the hβ-ala substitution at this site caused the peptide to revert to an activator form in the AIP-I group response, while retaining its cross-group inhibitory activity in other groups. Tal-Gan et al. (2013b) also created a highly potent global inhibitor with a D4A substitute (Fig. 2). The Asp-4 in AIP-III is adjacent to the third cysteine residue, which is involved in the thiolactone bridge, suggesting an importance for this position in specific AIP recognition as is the case of AIP-I. D4A-AIP-III also shows QQ activity *in vivo* at pico- or nanomolar concentrations, which attenuates hemolysis and the production of Toxic Shock Syndrome Toxin-1 (TSST-1). These virulent factors are known as outputs of the *agr* QS system in staphylococci (Janzon and Arvidson 1990; Novick et al. 1993, 1995; George and Muir 2007). Tal-Gan et al. (2013b) also synthesized a truncated AIP-III with modifications of D4A to F5Y or F5W, which were shown to be strong agonists for all AgrC receptors in *S. aureus* and significantly inhibited hemolysis against all AIP-I-IV strains *in vivo*.

*N*-methyl (*N*-Me) and peptoid (n) scanning was performed in order to understand SARs and the development of QQ peptides that target AIPs, like the AgrC interactions in *S. aureus* (Fowler et al. 2008; George et al. 2008; Tal-Gan et al. 2014). In this strategy, each side chain of a specific residue in AIPs is shifted from the C-α to an amide. For example, George et al. (2008) used an *N*-Me scan of the truncated macrocycle of AIP-II (tAIP-II) and discovered that H-bonds were essential for cognate and non-cognate AgrCs inhibition. In another study, a peptoid–peptide hybrid of the truncated AIP-I was found to be capable of modest inhibition of AgrC-I (Fowler et al. 2008). Tal-Gan et al. (2014) synthesized seven *N*-Me AIP-III analogues and six peptoid AIP-III analogues and their QQ activities were evaluated against cognate (AgrC-III) and non-cognate (AgrC1, II and IV) receptors. *N*-Me modifications of Cys-3 and Asp-4, and peptoid modifications to the Asp-4 macrocyclic ring of AIP-III, caused modest to large reductions in inhibitory activity relative to AIP-III, indicating the significance of the Cys-3 and Asp-4 amide NHs and the Asp-4 side chain position for certain non-cognate receptor interactions. A peptoid version of Phe-5, AIP-III nF5, produced the most potent inhibitor with an $IC_{50}$ value of 839 pM against AIP-IV. Further modifications to Leu-6 and Leu-7 in AIP-III abolished inhibitory activity and suggested that these residues are typically responsible for binding to non-cognate receptors (Tal-Gan et al. 2014).

It is worth mentioning about antibody dependent AIP inhibitors. Monoclonal antibodies were developed to target AIP-IV (Park et al. 2007). An isolated hapten-linked AIP-4 (AP4-24H11) was incubated with a wild type AIP-IV strain (RN4850) and group I strain (Wood 46). Results suggest that AP4-24H11 attenuated α-toxin production in both strains and also significantly abolished the severity of dermonecrosis in a skin and soft tissue infection mouse model when incubated with strain RN4850. Therefore, this study indicates that there is promise in the therapeutic use of antibodies against staphylococcal infections, although it has limitations due to its specific activity against specific strain types.

Broderick et al. (2014) used a model water-soluble polymer (carboxymethylcellulose) and D4A-AIP-III. The study demonstrated that thin layer coating of such polymer may allow rapid releasing of QSI in surrounding aqueous environment and could strongly modulate QS mediated virulent phenomenon of group-III *S. aureus*. Subsequently, Kratochvil et al. (2015) also

design nanoporous superhydrophobic coatings over D4A-AIP-III that promote the extended release of water-labile QSI and can be release into surrounding media for periods of at least 8 months. The study is found that coated this QSI can strongly inhibited QS mediated virulence phenomenon upto 40 days.

## 3.2 ZBzl-YAA5911 Targeting *fsr* System of *E. faecalis*

Enterococci are a common member in the normal intestinal flora of humans and animals. However, nowadays, antibiotic-resistant enterococci are highly common as they are similar to staphylococci and frequently cause infections (Marothi et al. 2005; Kristich et al. 2014). Notably, VRE is problematic and is not sensitive to antibiotics (Murray 2000). Among known virulence factors in enterococci, the gelatinase of *E. faecalis* is regulated by an *agr* like QS system, termed *fsr* (Nakayama et al. 2001). A lactone peptide named gelatinase biosynthesis activating pheromone (GBAP), consisting of 2 exocyclic amino acids and 9 macrocyclic moiety amino acids, mediates signaling for the *fsr* QS system (Nakayama et al. 2001). GBAP is translated from *fsrD* and then maturated by FsrB, which performs cyclization simultaneously with proteolytic cleavage of FsrD (Nakayama et al. 2006). GBAP interacts with the FsrC-FsrA two-component regulatory system corresponding to the AgrC-AgrA from the staphylococcal agr system, and eventually induces the transcription of the *gelE-sprE* operon, which encodes the gelatinase and serine proteases (Nakayama et al. 2001, 2006).

Unlike the staphylococcal *agr* system, no variants of AIPs have been found in *E. faecalis* so far, thus suggesting high conservation of the *fsr* QS gene cluster in *E. faecalis*. The lack of natural antagonists has led to de novo designs of GBAP antagonists. Furthermore, the SAR of staphylococcal AIPs is not fit with GBAP since tail amino acid residues did not result in remarkable changes in activity when they were replaced with alanine residues (Nishiguchi et al. 2009). Alanine scanning in the ring region revealed two critical residues, Phe-7 and Trp-10, which are indispensable for agonistic activity. Loss of the receptor binding activity of F7A-GBAP and W10A-GBAP indicated that these two aromatic residues are crucial for the receptor binding of GBAP (Nishiguchi et al. 2009). Unfortunately, any alanine substitutes showed GBAP antagonist activity (Nishiguchi et al. 2009). Thus, a novel reverse alanine scanning approach was taken to create GBAP antagonists (Fig. 3).

In the reverse alanine scanning strategy, a receptor binding scaffold (RBS) was created by substituting all residues except for the tail amino acids and two essential aromatic amino acids in the ring (Phe-7 and Trp-10 to alanine) (Nakayama et al. 2013). RBS showed very weak but significant QS inhibitory activity (25 % inhibition at 100 μM). For the next step, the substituted alanines in RBS were reverted to the original GBAP amino acids one by one while checking the inhibitory activity of each revertant. After three cycles of this reverse alanine scanning, [Ala5,9,11]-Z-GBAP (Z- benzyloxycarbonyl) was obtained as the maximally reverted peptide (MRP) with the strongest QSI activity (IC50 = 8.7 μM) among the series of revertants (Nakayama et al. 2013). The results indicate that Asn-5, Gln-9, and Met-11 were critical for the agonist/antagonist decision. Since Asn-5 was found to be the most critical residue for the antagonist activity among these three residues, it was substituted with different amino acids. Consequently, ZBzl-YAA5911 ([Tyr(Bzl)$^{5}$, Ala$^{9,11}$]Z-GBAP, Bzl- benzyl) was obtained as the most potent GBAP antagonist ($IC_{50}$ = 26 nM and Kd = 39 nM) (Fig. 3). The efficacy of ZBzl-YAA5911 was demonstrated in vivo by using an aphakic rabbit endophthalmitis model. It is known that the gelatinase induced by the fsr QS is involved in the translocation of E. faecalis from the aqueous humor into the vitreous cavity. ZBzl-YAA5911 significantly suppressed the translocation of E. faecalis and reduced retinal damage (Nakayama et al. 2013).

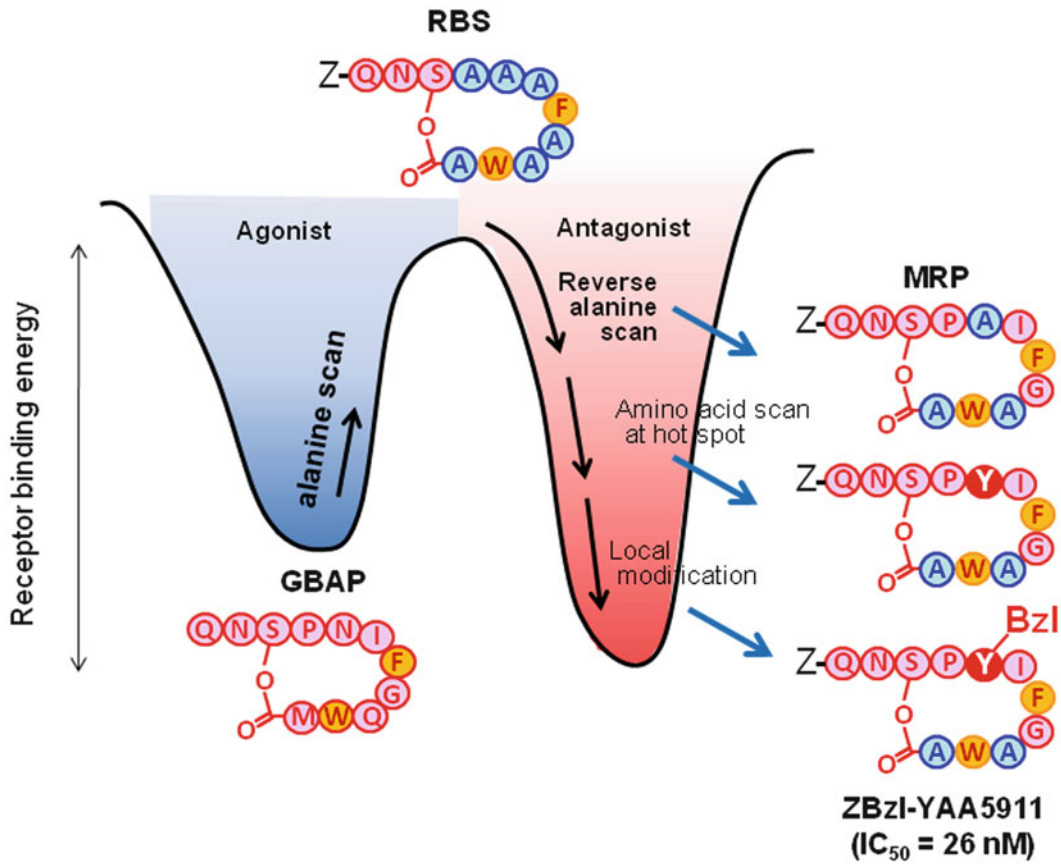


**Fig. 3** *de novo* design of the gelatinase biosynthesis activating pheromone (GBAP) antagonist by using the reverse alanine scanning approach. Alanine scans on GBAP did not derive an antagonistic peptide, suggesting difficulty to transit the receptor-binding energy summit between agonist and antagonist by using GBAP as a template. Thus, a reverse alanine scanning approach was taken. Receptor binding scaffold (RBS), which maximally substitutes alanine and is expected to have the lowest binding energy for the receptor, was initially created and then the alanines were reverted to the original amino acid one-by–one in order to enhance antagonist activity. Consequently, maximally reverted peptides (MRP) were obtained. Ala-5 in MRP was the most critical residue for antagonistic activity and was then substituted with tyrosine to enhance its antagonist activity. Finally, the tyrosine residue was modified and the most potent antagonist (ZBzl-YAA5911) was obtained with an $IC_{50}$ of 26 nM

## 3.3 AIP Antagonists Targeting VirSR System of *C. perfringens*

Anaerobic pathogenic *C. perfringens* is a spore forming Gram-positive bacterium that causes several diseases in humans and animals. It is the causative agent for gas gangrene (also called myonecrosis) (Hatheway 1990), necrotic enteritis, and food poisoning (Rood 1998; Uzal and McClane 2011). *C. perfringens* produces several extracellular enzymes and toxins (Lyristis et al. 1994; Shimizu et al. 1994). More generally, this bacterium produces four types of toxins, namely alpha-, beta-, epsilon-, and iota-toxins (Li et al. 2013). Clinical and environmental isolates of *C. perfringens* can be divided into five biotypes (from A to E) based on the types of toxins that they produce. The complete genome sequence and functional analyses have revealed that the *agrBD* locus is involved in the two-component regulatory system VirSR, termed the VirSR-TCS (Shimizu et al. 2002; Ohtani

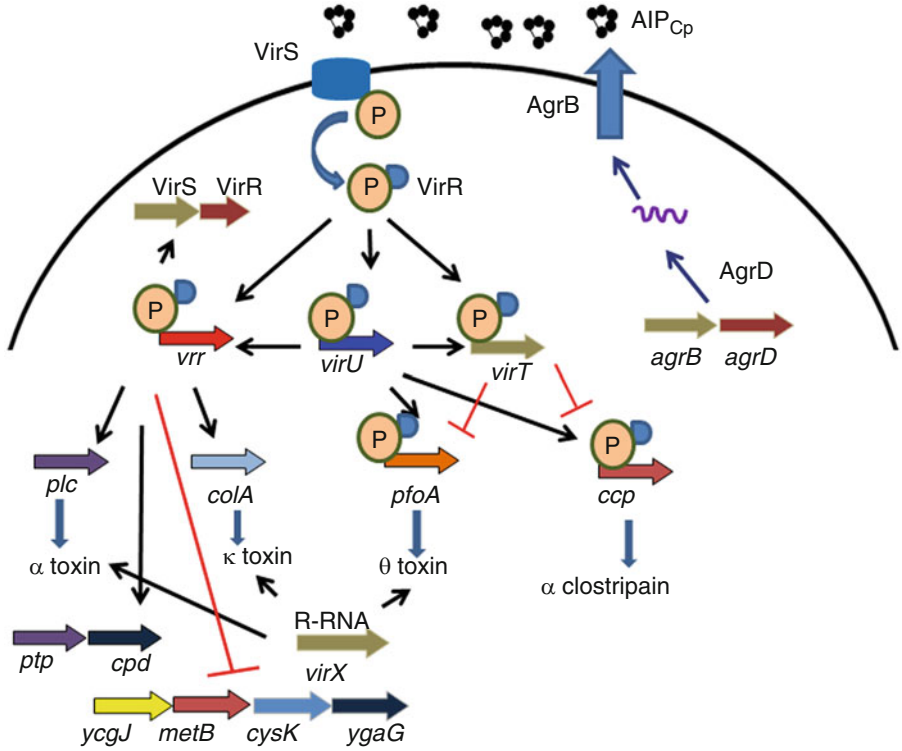


**Fig. 4** Schematic representation of the *agr* QS system in *Clostridium perfringens* type-A. VirSR-TCS positively regulates the production of α, θ, and κ toxins and extracellular enzymes. The VirS sensor is a histidine kinase and undergoes autophosphorylation in a cell density-dependent manner and ultimately induces phosphorylation of VirR. The phosphorylated VirR response regulator then recognizes and binds independently to two repeated sequences, called the VirR boxes, which are located up-stream of the target genes and three regulatory RNAs (*vrr*, *virU,* and *vir T*). These regulatory RNAs either positively or negatively control the expression of toxic genes and enzymes. The *vrr* is controlled the production of the VirS and VirR proteins. The VirRS-TCS is also reported to induce the expression of *cpd* (encoding 2', 3'-cyclic nucleotide phosphodiesterase), *ptp* (encoding protein tyrosine phosphatase), and *hyp-7* (regulatory RNA), and it suppresses the expression of the *ycgJ-metB-cysK-ygaG* operon

et al. 2009, 2010). The *agrBD* locus is different from the *agrBDCA* and *fsrABDC* locus present in *S. aureus* and *E. faecalis*, respectively, in which the genes required for the receptor (VirS) and response regulator (VirR) for AIP$_{Cp}$ are encoded at a different locations of the genome region (Ohtani et al. 2009, 2010). Similar to *S. aureus*, the pro-AIP produced by agrD is further processed and transported by AgrB (Fig. 4). The VirS sensor is a histidine kinase and undergoes autophosphorylation in a cell density dependent manner, ultimately inducing the phosphorylation of VirR (Ohtani et al. 2009). The phosphorylated VirR response regulator then recognizes and binds independently to two directly repeated sequences, called the VirR boxes, which are located up-stream of the target genes (Fig. 4). It is known that only two toxin genes (*pfoA* and *ccp*) and three regulatory RNAs (*vrr, virU and virT*) have VirR binding sites (Shimizu et al. 2002). Therefore, the regulation of enzymes and toxins in *C. perfringens* is a complex system but necessary for host tissue digestion and nutrient uptake for cellular metabolism and biosynthesis as well as for the establishment of severe gangrenous infection (Shimizu et al. 2002). Therefore, VirRS-TCS represents a suitable target site for developing antipathogenic strategies using QSI for attenuating virulence and minimizing bacterial resistance.

On the basis of the deduced amino acid sequences of AgrD, now 5-membered cyclic peptide (CLWFT) has been confirmed as an $AIP_{Cp}$ in *C. perfringens* where the thiolactone ring occurs between 1st and 5th residue (Ma et al. 2015). However, this peptide has also been identified from *C. perfringens* strain 13 (type A) culture filtrates in our laboratory (Singh et al. 2014). The Ma et al. (2015) study used four different types (A, B, C and D) of *C. perfringens* strains and found that all four strains can recognize a single type of peptide without autoinducer interference as reported in the case of *S. aureus* (Ji et al. 1997). This particular study also synthesized 6 membered thiolactone macrocyclic peptides, including CLWFTH, which competitively inhibited beta toxin (CPB) production in *agrB* null mutants of CN1795 (type B) and CN3685 (type C). Nevertheless, we rationally designed and synthesized potent QQ peptides following $AIP_{Cp}$ - VirS activity (Singh et al. 2015). The study has found that Trp-3 and Phe-4 are responsible for receptor recognition and activation respectively. Substitution of The-5 with alanine or serine has been confirmed that free methyl group The-5 is also important for receptor activation. Based on structure-activity relationship, we have designed two potent QQ compounds, Z-AIPCp-L2A/T5A (partial agonist) and Z-AIPCp-F4A/T5S (partial antagonist). These peptides significantly inhibited transcription of pfoA with $IC_{50} = 0.32$ and 0.72 μM, respectively.

## 4 RNA III Inhibiting Peptides (RIPs) to Prevent Staphylococci Biofilm Formation

The RAP-TRAP (RAP-targeting of the RNAIII-activating peptide) signal transduction system is one of those regulators that controls the production of TSST-1, enterotoxins, proteases, and δ-hemolysins, which allows *S. aureus* to survive, disseminate, and establish an infection (Balaban et al. 2001). Thus, complex regulatory networks associate with the *agr* QS system (Bronner et al. 2004). RAP is a six residue peptide (YKPITN) and activates the *agr* QS system via phosphorylation of TRAP (Balaban et al. 2000b). Gov et al. (2001) suggested that RAP increases cell adhesion and biofilm formation in addition to *agr* activation. Furthermore, it was found that RIP, which is a heptapeptide (YSPXTNF, where X can be a Cys, a Trp, or a modified amino acid) that was originally isolated from culture supernatants of coagulase negative staphylococci (later identify as *Staphylococcus xylosus*), efficiently prevents biofilm formation both *in vivo* and *in vitro* and is especially associated with catheter infections (Balaban et al. 2001). Further research found that a synthetic RIP (YSPWTNF) was extremely effective in inhibiting RNA III expression *in vitro* and concurrently inhibited *S. aureus* strains I-IV infections *in vivo*, including keratitis, cellulitis, osteomyelitis, septic arthritis, and mastitis (Balaban et al. 2000a).

In fact, RAP- TRAP QS system has been proposed as master regulator of modulating several virulent genes by *agr* system (Cirioni et al. 2006; Balaban et al. 2007). Several studies have initially raised a controversy against this system (Shaw et al. 2007; Tsang et al. 2007; Novick and Geisinger 2008). For example, the studies of Shaw et al. (2007) demostrated that TRAP protein is not involved in modulating virulence gene expression or biofilm formation, and has no effect on the Agr QS system in *S. aureus*. The study did not find out any impairment in gene expression levels of multiple *agr*-regulated genes (*hla, sspA*, and *spa*). However, using independently of RIP or with the combination of antibiotics *in vivo* model systems has been postulated that blockage of RAP-TRAP interaction is an important site for minimizing drug-resistant *S. aureus* infection (Dell'Acqua et al. 2004; Domenico et al. 2004; Cirioni et al. 2006; Lopez-Leban et al. 2010) and this system may work parallel to *agr* QS system *in vivo*. It is also noted that the molecular mechanism of this interaction is not fully known yet.

It was assumed that TRAP sequences are highly conserved between different strains, which are different from those of the *agr* QS genes. Indeed, the RIP effect is independent of the *S. aureus* strains. Since then, several RIP analogous have been synthesized and have a

consensus sequence, YXPXTNX, in which X can be replaced with other amino acids while maintaining the RNA III inhibiting activity, but only YKPITNF and YSPWTNF were effectively shown to inhibit infections *in vivo* (Gov et al. 2001). Gov et al. (2001) also synthesized a high temperature-stable RIP, YSPWTNF-amide, which significantly inhibited RNAIII synthesis at 5 μg RIP/$10^6$ *S. aureus* cells. A structure–activity study of RIP was performed using alanine scanning and truncation of YSPWTNF revealed that Thr-5 is essential for RIP activity while the truncated tetra-peptide, SPWT, has the best anti-staphylococcal activity (Baldassarre et al. 2013).

Attenuation of virulence gene expression in different drug-resistant strains of *S. aureus* using RIP has been evaluated, such as MRSA, glycopeptide-intermediate and vancomycin-intermediate (Giacometti et al. 2005). Central venous catheter (CVC) associated streptococcal infections can be significantly reduced using pre-treatment with RIP, followed by antibiotic drugs. For instance, Cirioni et al. (2006) pre-treated CVC with RIP (10 μg in 10 μL of Mueller-Hinton broth) then added antibiotics (ciprofloxacin, imipenem, and vancomycin) but found no difference in the minimum inhibitory concentrations (MIC) and minimum biofilm inhibition concentrations (MBIC) against planktonic cells, but MICs and MBICs against biofilms were drastically reduced. Thus, the combination of RIP and polymethylmethacrylate beads can be used to control orthopedic infections caused by MRSA ATCC43300 biofilms (Anguita-Alonso et al. 2007). It was also reported that treatment of *S. aureus* graft infected mice with RIP 2 days post-infection suppressed bacterial growth and was more efficacious with multiple doses (Balaban et al. 2007). Moreover, it was demonstrated that RIP treatments prevented *Staphylococcus xylosus* biofilm development *in vitro* and *in vivo* (Balaban et al. 2003).

Antibody therapies that target the RAP-TRAP system were also investigated. RNAIII synthesis can be directly inhibited by antibodies against either RAP or TRAP (Leitner et al. 2011). Mice vaccinated with RAP do not succumb to *S. aureus* infections (Balaban et al. 1998). A recombinant TRAP (rTRAP) vaccine was developed and was found to be safe when administered as 2–3 subcutaneous injections at 54–100 μg with the adjuvant ISA 206 against staphylococcal mastitis (Leitner et al. 2011). Furthermore, this treatment did not lead to any abnormal symptoms of sensitivity to the vaccine in cows and goats. The rTRAP vaccine caused the induction of a humoral immune response, which remained high for at least 160 days post second immunization (Leitner et al. 2011). The protection level was high with the titer of anti-RAP antibodies and rTRAP vaccination, indicating that these are promising vaccine candidates against staphylococcal infection.

## 5 Signal Peptidase Inhibitors to Block AIP Biosynthesis in Staphylococci

The AIP biosynthetic pathway is a fascinating target for the development of inhibitors for the QS system. The AIP precursor, AgrD, of staphylococci and enterococci was processed and cyclized to the mature form by AgrB/FsrB (Nakayama et al. 2001; Zhang and Ji 2004; Qiu et al. 2005). It is known that AgrB and SpsB are involved in this process in the case of *S. aureus,* but this mechanism has not yet been completely elucidated (Fig. 1). Deduced amino acid sequences suggest that AgrB localizes to the cell membrane and that it acts as a cysteine protease and a transporter for mature AIPs (Zhang and Ji 2004). Two residues (His-77 and Cys-84) were identified as being essential for proteolytic activity, suggesting that AgrB acts as a cysteine protease (Qiu et al. 2005). These two residues are highly conserved in every AgrB-like protein, including those in non-staphylococcal species, which highlights their important function. The C-terminal extension tail in pro-AgrD is removed by the protease activity of AgrB concomitantly with cyclization, while the N-terminal amphipathic helix is removed by a chromosomally encoded signal peptidase, termed SpsB (Kavanaugh et al. 2007).

Kavanaugh et al. (2007) successfully designed peptide inhibitors of SpsB, which mimicked the

sequence around the N-terminal cleavage site of AgrD. Two designed peptides, NIIFKPST-amide and NIAAPST-amide, both having a proline at the +1 position of the cleavage site, significantly inhibited *agr* QS by interfering with the peptidase activity of SpsB. However, millimolar concentrations were necessary for QQ activity. The stronger peptide, NIIFKPST-amide, showed growth inhibitory activity at concentrations above the QS inhibitory range (Kavanaugh et al. 2007). Thus, it appears that SpsB is not only involved in AIP biosynthesis but also secretes a number of other secreting proteins, some of which appear to be crucial for growth.

## 6 Natural Interference of QS Among *Staphylococcus* Species and Other Genera

AIPs of *Staphylococcus epidermidis* and *S. lugdunensis* have shown antagonistic activity for the AIP of *S. aureus* in addition to the QS interference of *S. aureus* strains (Otto et al. 1999). Such cross-inhibition among different AIP groups is referred to as bacterial interference. Cross genera QS interference has also been reported, in which *Lactobacillus reuteri* RC-14 attenuated the ability of *S. aureus* to cause toxic shock syndrome through the production of cyclic dipeptides like cyclo(L-Tyr-L-Pro) and cyclo(L-Phe-L-Pro) (Li et al. 2011). Competition assays indicated that these dipeptides may compete for the ligand-binding pocket on the AgrC receptor (Li et al. 2011). Moreover, the long chain acyl homoserine lactone (AHL, 3-oxo-12-HSL) produced by *Pseudomonas aeruginosa* not only acts in the QS processes for this bacterium but also cross-inhibits the *agr* base QS in *S. aureus* with an $IC_{50}$ of 6 μM (Qazi et al. 2006). Murray et al. (2014) synthesized a series of 3-oxo-$C_{12}$-HSL, tetramic acid, and tetronic acid analogues to gain insights into the structural requirements of AHL molecules for the *agr* inhibition. As a result, it was found that the compounds were noncompetitive inhibitors of AIP and altered the activation efficacy of AIP like allosteric modulators. In addition, the efficacy of the most potent 3-tetradecanoyltetronic acid was demonstrated in a mouse infection model. This compound either prevents AgrC receptor dimerization or interferes with the interactions between AgrC and AgrA, subsequently inhibiting P3 activity in *S. aureus* (Murray et al. 2014). *P. aeruginosa* also produces oxidized quinoline that disturbs AIP signaling by destabilizing cytoplasmic membrane proteins (Mitchell et al. 2010). Peterson et al. (2008) found that cross-kingdom QS interference in mice models where mice serum containing apolipoprotein (ApoB) bound to the AIP of *S. aureus* and attenuated *agr* QS signaling. ApoB100 produced by human livers and ApoB484 produced by the liver and enterocytes as chylomicrons act as inhibitors of *agr*-signaling with $IC_{50}$ values of 2.3 and 3.5 nM, respectively (Elmore et al. 2015).

## 7 Screening QQ Compounds from Natural Resources

Several QQ compounds have also been reported to quench the QS system in bacterial pathogens and some of the best examples have been summarized below. An extensive random screening program identified several synthetic and natural small molecule analogues with *agr* inhibitory activity (Sklar and Gresham 2011; Sully 2011). Although the mechanism of action for these compounds (below) has yet to be elucidated, they were subjected to *in vivo* mouse model experiments and their anti-infection efficacies were demonstrated. Notably, savirin and benzbromarone inhibited AIP-induced RNAIII production at submicromolar concentrations, especially savirin inhibited this production in all four *S. aureus agr* groups except for the important skin commensal, *S. epidermidis* strain 12228 (*agr* group 1) (Sklar and Gresham 2011; Sully et al. 2014). The mechanism of inhibition by savirin is mediated solely by interfering with AgrA binding to DNA (Sully et al. 2014). Khodaverdian et al. (2013) has prepared a Nested Chemical Library of small compounds and found that the drug diflunisal targets the phosphoryl binding pocket of AgrA and interferes with AgrA- DNA

binding activity at the P3 site in the MRSA strain USA300. A polyhydroxyanthraquinone global inhibitor compound, ω-hydroxyemodin (OHM), was isolated from solid-phase cultures of *Penicillium restrictum* and shown to inhibit the QS signaling of all four groups of *S. aureus* (Daly et al. 2015). Furthermore, the study demonstrated that the compound directly bound to AgrA and prevented the interaction of AgrA with the *agr* P2 promoter. It decreased dermonecrosis and reduced inflammatory cytokine transcription and expression at the site of infection in the OHM treated mice model system. OHM also enhanced the immune cell mediated killing of *S. aureus in vitro* in an *agr*-dependent manner. Another compound, colostrum hexasaccharide, was found to inhibit the activities of several virulence factors (spreading ability, hemolysis, protease, and lipase) associated with *S. aureus* infections (Srivastava et al. 2015).

Screening natural QSIs using more than five hundred marine bacterial strains identified six novel cyclodepsipeptides from the marine Gram-negative bacterium *Photobacterium*, solonamide A/B and ngercheumicins (F, G, H, and I), as *agr* QS inhibitors (Mansson et al. 2011; Kjaerulff et al. 2013). The structural similarity suggests that these compounds may function as competitive inhibitors of *agr*C in *S. aureus* (Mansson et al. 2011). In another study, a cyclic peptide (Avellanin C) obtained from the fungus *Hamigera ingelheimensis* attenuated the QS system of the *S. aureus agr* reporter strain (8325–4) with an $IC_{50}$ value of 4.4 μM (Igarashi et al. 2015). Phytochemicals are also good candidates for QQ compounds. The group of Wang J found a number of phytochemicals (such as chrysin, α-cyperone), some of which were found in Chinese herbs, to effectively suppress the alpha-haemolysin production in *S. aureus* via *agr* QS inhibition in mouse model (Qiu et al. 2011; Wang et al. 2011; Luo et al. 2012).

Kiran et al. (2008) identified 2, 5-di-O-galloyl-d-hamamelose (hamamelitannin) as a nonpeptide analog of RIP by virtual screening of a RIP-based pharmacophore. Hamamelitannin is a natural compound that was originally obtained from the bark of *Hamamelis virginiana* (witch gazel) and does not affect growth of *S. aureus* (Wang et al. 2003). It inhibited production of RNAIII and subsequently prevented biofilm formation and cell attachment under *in vitro* conditions (Kiran et al. 2008). Furthermore in a rat graft model, hamamelitannin attenuated virulent gene expression in methicillin-resistant *S. aureus* and *S. epidermidis* strains from a device-associated *in vivo* condition. The combination of QQ agents and antibiotics is also suggested as a novel strategy to combat MRSA strains. An adjuvant of 1 μg $ml^{-1}$ three biaryl hydroxyketones (F1, F12, or F19) reduces the MIC of nafcillin and cephalothin about 50-fold to values comparable to those of vancomycin in murine models (Kuo et al. 2015).

From secondary metabolites of actinomycetes, Nakayama et al. (2007) identified siamycin as a QSI of the *E. faecalis fsr* QS system, which is a tricyclic peptide antibiotic of *Streptomyces* and is also known as an anti-HIV peptide agent. Siamycin inhibited QS-induced gelatinase production at 10 nM but it did not affect bacteria growth. It disturbed the signal transduction of the FsrC-FsrA two-component regulatory system in a noncompetitive manner (Nakayama et al. 2007). It was also demonstrated that siamycin inhibited autophosphorylation of the histidine receptor kinase, FsrC (Ma et al. 2011; Phillips-Jones et al. 2013).

Nakayama et al. (2009) also identified ambuic acid as an anti *fsr* QS compound, which was isolated from fungal metabolites. Bioassay of this study indicated that ambuic acid blocked the biosynthesis of GBAP through the inhibition of FsrB function. FsrB belongs to the AgrB protein family, which is widely conserved among Gram-positive bacteria belonging to phylum *Firmicutes*. His-77 and Cys-80 in FsrB function as catalytic residues in the cysteine protease, are involved in AIP processing, and are completely conserved among the AgrB family of proteins found in the genome sequence database (Nakayama et al. 2009). This suggests that the molecular mechanism of enzymatic reaction in AIP biosynthesis is common among the thiolactone/lactone autoinducing peptides encoded in the genome of high GC content

Gram-positive bacteria and that QSI targeting of the AgrB protein family may commonly inhibit the biosynthesis of those thiolactone/lactone AIPs. Indeed, ambuic acid inhibited the biosynthesis of AIP in *S. aureus* and *Listeria innocua* as well as GBAP (Nakayama et al. 2009). Hence, QQ targeting of peptidase function in the AgrB protein family would be fascinating in terms of gaining a broad spectrum that can act as an effective QSI.

Recently, our group (Desouky et al. 2013; Shojima and Nakayama 2014) has established a high-throughput system (HTS) for screening QSI compounds that target the thiolactone/lactone peptide-mediated QS of Firmicutes. The system consists of three screening steps, including the first two steps of an anti *agr* QS screening protocol using dual-reporter strains of *S. aureus* and the last step of an anti *fsr* screening protocol using a conventional *E. faecalis* gelatinase assay. By using this HTS system, it was expected to provide a broad spectrum QSI that targets the cyclic peptide-mediated QS in Gram-positive pathogens. Subsequently, our recent study (Desouky et al. 2015) found three cyclodepsipeptides (WS9326A, WS9326B, and cochinmicin II/III) in culture extracts of actinomycetes. These molecules were known to be receptor antagonists, tachykinin (WS9326A and WS9326B) (Hayashi et al. 1992; Kino et al. 1993) and endothelin (cochinmicin II/III) (Lam et al. 1992; Zink et al. 1992). All three compounds were found to be antagonists against the FsrC of *E. faecalis*, while WS9326A and WS9326B significantly attenuated hemolysis in the case of *S. aureus* strains 8325–4 (type-I AIP), K12 (type-II AIP), and K9 (type-IV AIP). Interestingly, WS9326A also inhibited the transcription of the *pfoA* toxin in *C. perfringens* - 13 (type-A) with an $IC_{50}$ of 0.88 μM. Additionally, an *ex vivo* assay was performed to examine the efficacy of WS9326B to attenuate the toxicity of Newman (type-I) and K3 (type-II) *S. aureus* strains in corneal epithelial cells (Desouky et al. 2015). As a result, WS9326B greatly decreased the cytotoxicity of these *S. aureus* strains at 20 μM by suppressing the production of toxins, causing keratitis.

## 8 Future Perspective

Pathogenic bacteria are often resistant to traditional antibiotics and the native immune system, therefore, we are forced to develop new strategies to control infectious diseases. From the past couple of decades, we understand many things due to the advancement of molecular tools that allowed us to develop a deeper understanding of the molecular and chemical basis of bacterial pathogenicity. Given this understanding, we learned that the expression of certain genes, including virulent ones from pathogenic bacteria, depends on a well-coordinated signaling system that is modulated by small molecules and can be attenuated without disrupting cell viability. In the case of Gram-positive bacteria, a ligand-based design of competitive AgrC inhibitors through alanine scanning has provided compelling proof that virulence gene expression can be inhibited through chemotherapeutic interference. After understanding the SAR of AIPs (AgrC/FsrC), a number of the AIPs analogues synthesized that displayed an $IC_{50}$ at nanomolar or sub-nanomolar micromolar concentrations are stable and can suppress pathogenicity *in vivo*. Simultaneously, several natural QSIs have been identified, which disrupt the QS system via interaction to different sites. The development of QSIs that target *C. perfringens* is in the juvenile stage but the information generated through *S. aureus* and *E. faecalis* will significantly increase our chances of finding a potent inhibitor that targets the VirS receptor in order to fight this notorious pathogen. Further developed strategies will spot light on interference and overlapping between investigated *agr*/*agr*-like QS systems which will be useful for well understanding of virulence gene regulation.

It is noticed that even QQ strategy has a risk to urge the emergence of drug resistant strains (Defoirdt et al. 2010; Maeda et al. 2012; Garcia-Contreras et al. 2013; Garcia-Contreras et al. 2015). It was demonstrated that bacterial resistance arises rapidly to a best-characterized compound (brominated furanone C-30) that inhibits acyl homoserine lactone mediated QS system due to mutations that increase the efflux

of this QQ molecules. It was also suggested that QQ therapy might impose a high immune selective pressure on the core gene(s) of QS system, that would promote the growth of QQ resistant bacterial strains (Defoirdt et al. 2010; Garcia-Contreras et al. 2015). On the other hand, it was reported that savrin that blocks the *agr* QS system through the specific inhibition of the AgrA-promoter interaction in *S. epidermidis* did not lead to emerging of QQ resistant strains while promoting host defense system (Sully et al. 2014). This example suggests the importance and possibility that QQ therapy should not generate selective pressures for antibiotic-resistant strains but should provide sufficient time to activate host immune defense systems in order to permanently remove the pathogens (Amara et al. 2011). Therefore, we definitely require nontoxic compounds targeting specific site with enough low $IC_{50}$, far from the dose showing pleiotropic effect. There is a good example in which an AIP antagonist (AIP-III D4A, Tal-Gan et al 2013b) with sub-nanomolar $IC_{50}$ was successfully applied to polymer coating, which strongly inhibit *agr*-based QS of *S. aureus* on the surface for at least 40 days (Kratochvil et al. 2015). AIP antagonists would be advantageous because they are expected to specifically interact with the receptor and not with the other molecules, that make bacteria free from any toxic stress. With ever-increasing drug design and delivery technologies, rationale design of AIP antagonist will be pursued to create ideal QQ molecules as post-antibiotic agents.

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