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Gianfranco Donelli *Editor*

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In Vitro Activity of Tigecycline Against *Acinetobacter baumannii*: Global Epidemiology and Resistance Mechanisms

Spyros Pournaras, Vasiliki Koumaki, Vasiliki Gennimata, Evangelia Kouskouni, and Athanassios Tsakris

Abstract

Acinetobacter baumannii is a pathogen of increasing concern, commonly causing outbreaks in the hospital environment. Of particular concern, *A. baumannii* strains exhibiting resistance to carbapenems, which were previously considered the treatment of choice for infected patients, have dramatically increased worldwide, leaving a few antibacterial choices. Tigecycline, a broad-spectrum modified minocycline derivative, isconsidered as a last resort drug against multidrug-resistant *A. baumannii*. Though, resistance to tigecycline has emerged and is growing notably following increasing tigecycline usage. Comparative evaluation of the tigecycline resistance rates reported worldwide is challenging due to the absence of official interpretative criteria for in vitro susceptibility testing and the discrepancies among the different susceptibility methodologies used, with broth microdilution being considered the reference method. Tigecycline resistance is mainly associated with resistance-nodulation-cell division (RND)-type transporters, mainly the AdeABC, AdeFGH and AdeIJK efflux pumps, but other resistance mechanisms have also been implicated. Tigecycline is still an attractive choice for *A. baumannii*, but further investigations are warranted so that treatment of MDR A. *baumannii* could be guided by validated *in vitro* data.

Keywords

Tigecycline • *Acinetobacter baumannii* • Resistance • MIC • Resistance mechanisms • Susceptibility methods

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1 Introduction

Acinetobacter baumannii complex has emerged as one of the most important pathogens especially in nosocomial environments and intensive care units (ICUs) (Lin and Lan 2014). *Acinetobacter baumannii* (formerly named genomic species 2), *Acinetobacter pittii* (formerly named genomic species 3) and *Acinetobacter nosocomialis* (formerly named genomic species 13TU) (Nemec et al. 2011) represent the most pathogenic *Acinetobacter* species for humans. These three pathogenic species along with the environmental species *Acinetobacter calcoaceticus*, which has been recovered from soil and water (Towner 2009), but with minor clinical relevance have been designated as *A. calcoaceticus-baumannii complex* (Doi et al. 2015). They present similar phenotypic profile, which does not allow manual and semi-automated commercial routine identification methods to distinguish among them (Higgins et al. 2007). The precise identification of *Acinetobacter* isolates to species levels is challenging and requires genotypic methods, such as amplified 16S ribosomal DNA restriction analysis (ARDRA), tRNA spacer fingerprinting and selective amplification of restriction fragments (AFLP). Specific gene sequences can also be used, including intergenic spacer (ITS) region between the 16S and 23S rRNA genes, *rec*A, *rpo*B, and *gyr*B (Espinal et al. 2012). Lately, mass spectrometry has given the option of identifying isolates that belong to the *A. baumannii* group (consisting of the species *A. baumannii, A. pittii, A. nosocomialis*) (ECDC 2013).

A. baumannii is isolated mainly from the respiratory tract, bloodstream,, urinary tract, abdominal, skin, soft tissues and central nervous system (Spiliopoulou et al. 2014). *A. baumannii* is extremely resistant to desiccation and can survive on inanimate surfaces for a long time. It develops readily multidrug resistance by acquiring large resistance elements, called antibiotic resistance islands (Nigro and Hall 2012). The prior use of imipenem, meropenem, piperacillin/tazobactam or fourth-generation cephalosporins and >30 days of being bed-ridden are independent risk factors for extensively drug-resistant *A. baumannii* (XDRAB) infections (Chan et al. 2014; Pachon-Ibanez et al. 2004). Notably, the majority of nosocomial *Acinetobacter* isolates currently exhibit resistance rates to carbapenems as high as 80 % (ECDC 2013). It should be noted that carbapenems were widely used as last resort antibiotics for the treatment of severe infections, with carbapenem resistance to often leave few active antibiotic options. Among the available choices are most commonly included colistin and tigecycline (Sun et al. 2013), while in many cases minocycline remains also potent (Balode et al. 2013). However, tigecycline resistance in *A. baumannii* is a mounting concern. Tigecycline-resistant isolates have been recovered from patients treated with tigecycline (Hua et al. 2012; Hornsey et al. 2011), but also from patients that did received previously the drug (Deng et al. 2014; Sun et al. 2010).

Tigecycline is a modified tetracycline with a 9-t-butyl- glycylamido side chain added to the central skeleton of minocycline (Petersen et al. 1999), broadening its antimicrobial spectrum and rendering it active against multidrug-resistant (MDR) gram-positive and gram- negative, anaerobic and atypical bacteria (Peleg et al. 2007). Tigecycline inhibits the 30S ribosomal subunit and is capable to escape the tetracycline resistance mechanisms *tet(A)* to *tet(E)* and *tet(K)*, which encode efflux pumps and *tet(M)* and *tet(O)* that offer ribosomal protection (Fluit et al. 2005). Tigecycline has been approved by the FDA for complicated skin and skin-structure infections, complicated intra-abdominal infections, and community-acquired bacterial pneumonia (Stein and Babinchak 2013). However, tigecycline has been used for off-label indications, as a last resort for the management of infections mainly due to MDR Gram-negative bacteria.

2 Global Epidemiology of Tigecycline Resistance in *A. baumannii*

Susceptibility testing of tigecycline against *A. baumannii* has been problematic, since there are no established guidelines and many studies have been controversial. Tigecycline reaches low concentrations of 0.62–0.72 mg/L in serum,

(Karageorgopoulos et al. 2008) and undergoes extensive transfer from the blood into the tissues, where levels far exceed those of serum. For instance, its concentration in alveolar cells is 77.5-fold higher than in serum (Brink et al. 2010).

CLSI and EUCAST do not suggest breakpoints for tigecycline against *A. baumannii*. EUCAST reports the epidemiological cutoff (ECOFF) MIC value of tigecycline among *A. baumannii* to be 1 mg/L and the MIC wildtype distribution to range between 0.064 and 1 mg/L. BSAC, on the other hand redirects the researchers to EUCAST PK/PD non-species-specific breakpoints of S = 0.25 mg/L and R = 0.5 mg/L in order to interpret the results (BSAC 2015). However, so far, most of the researchers use the less strict breakpoints suggested by the FDA for *Enterobacteriaceae* (susceptible MIC ≤ 2 mg/L; resistant MIC ≥8 mg/L) or the EUCAST criteria for *Enterobacteriaceae* (susceptible MIC ≤ 1 mg/L; resistant MIC ≥ 2 mg/L).

Moreover, the *in vitro* activity of tigecycline against *A. baumannii* varies depending on the method used. E-test is reported to give increased MICs and therefore higher resistance rates than the broth microdilution method with FDA (Pillar et al. 2008; Thamlikitkul and Tiengrim 2008; Kulah et al. 2009), EUCAST (Grandesso et al. 2014) and BSAC criteria (Casal et al. 2009) used. It has been suggested that increased concentration of manganese in Mueller-Hinton agar results in increased MICs (Fernandez-Mazarrasa et al. 2009; Casal et al. 2009; Thamlikitkul et al. 2007) and smaller zone diameters (Thamlikitkul and Tiengrim 2008; Canigia and Bantar 2008), which could result in discrepancies when MHA from different manufacturers are used, or even among lots of the same manufacturer (Pillar et al. 2008). Fernandez-Mazarassa et al. consider that media with low manganese are more clinically relevant, since the concentration in human sera is low (0.8–1.2 μg/L) (Fernandez-Mazarrasa et al. 2009). However, other studies have shown excellent agreement between E-test and broth microdilution (Zarkotou et al. 2012). Agar dilution has shown acceptable minor errors compared to broth microdilution method (Zarate et al. 2010). Jones et al. proposed some modification of the tigecycline FDA disk diffusion breakpoints for *Enterobacteriacae* (≥19 mm sensitive/≤14 mm resistant) when applied to *Acinetobacter spp* (≥16 sensitive/≤12 mm resistant) (Jones et al. 2007). Application of these modified breakpoints, though, is controversial (Liao et al. 2008). Aged media have been accounted for increased MICs (Hope et al. 2005) because the activity of tigecycline is affected by the amount of dissolved oxygen leading to acceleration of oxidative degradation (Bradford et al. 2005).

The use of VITEK 2 in determining susceptibility of tigecycline against *A. baumannii* is also controversial. Leal Castro et al., reported that VITEK 2 was reliable with agreement up to 94 % (Leal Castro et al. 2010), while unacceptable errors have been reported elsewhere when VITEK 2 was used (Zarkotou et al. 2012; Grandesso et al. 2014). Piewngam et al., suggests that disk diffusion, E-test and VITEK-2 could be useful when breakpoints are adjusted, i.e. for disk diffusion ≥17 sensitive/≤12 resistant and MIC breakpoints S ≤ 1/R > 2 mg/L (Piewngam and Kiratisin 2014).

As previously reported, routine identification methods commonly used by laboratories in most regions cannot distinguish among the *Acinetobacter* complex, with non-*baumannii* species tending to present better sensitivity profiles (Chuang et al. 2011).

Worldwide studies of in vitro activity of tigecycline against *Acinetobacter spp*. report a wide range of non-susceptibility rates (Table 1). This could be due to a number of reasons, such as the small sample size examined, the possible clonal relationship between the isolates tested or the inclusion of only MDR *A. baumannii* isolates in some reports, which tend to exhibit higher resistance rates. Moreover, the identification and the susceptibility testing method used, the breakpoints adopted and the year of sample collection may also play a role.

Table 1 Worldwide reports of tigecycline resistance in Acinetobacter species

Reference	Pathogen	No of isolates	Region	Methodology	Susceptibility breakpoints	Non-susceptible rates %	Collection date
Navon-Venezia et al. (2007)	*Acinetobacter baumannii*	82	Israel	E-test	≤2, FDA	78	2003
Tan et al. (2007)	*Acinetobacter spp*	55	Singapore	Agar Dilution method	≤2, FDA	29	2004–2006
Kulah et al. (2009)	*Acinetobacter baumannii*	91	Turkey	BMD	≤2, FDA	14.3	2005–2007
Liao et al. (2008)	*Acinetobacter baumannii*	393	Taiwan	BMD	≤2, FDA	19.1	2006
Liu et al. (2008)	*Acinetobacter baumannii*	393	Taiwan	BMD	≤2, FDA	19.1	2006
Dizbay et al. (2008)	*Acinetobacter baumannii (MDR)*	66	Turkey	E-test		47	1/9/2006
Araj et al. (2008)	*Acinetobacter spp*	64	Lebanon	Disk diffusion	S ≥16 mm R ≤ 12	2	3/2006–12/2007
Teng et al. (2014)	*Acinetobacter calcoaceticus complex*	141	Taiwan	Disk diffusion	S ≥19 mm R ≤ 14	29	7/2006–6/2012
Behera et al. (2009)	*Acinetobacter baumannii (MDR)*	26	India	E-test	≤2, FDA	57.6	7–9/2007
Chang et al. (2012)	*Acinetobacter baumannii (MDR)*	141	Taiwan	BMD	≤2, FDA	45.5	2007
Kim et al. (2010)	*Acinetobacter spp (imipenem non-susceptible)*	190	Korea	Agar Dilution method	≤1 > 2, BSAC	23.4	2007
Al-Sweih et al. (2011)	*Acinetobacter spp (88.4 % MDR)*	250	Kuwait	E-test	≤2, FDA	13.6	5–12/2008
Taneja et al. (2011)	*Acinetobacter calcoaceticus- baumannii*	224	India	Disk diffusion	NS	14.2	2/07–6/08
Guven et al. (2014)	*Acinetobacter baumannii (MDR)*	145	Turkey	NS	≤2, FDA	RR 81	2008–2011
Van et al. (2014)	*Acinetobacter calcoaceticus- baumannii*	63	Northern Vietnam	VITEK-2	≤2, FDA	41.3	2009
Baadani et al. (2013)	*Acinetobacter spp*	1307	Riyadh, Saudi Arabia	VITEK-2	≤2, FDA	RR 9.7	2011
Jiang et al. (2014)	*Acinetobacter baumannii (MDR)*	42	China	WalkAway 96 PLUS NC50	N/A	40.5	12/2012–1/2013
Farrell et al. (2010)	*Acinetobacter spp*	397	Asia, W. Pacific	BMD	≤2, FDA	0.2	2008

Scheetz et al. (2007)	*Acinetobacter baumannii (carbapenemase non-susceptible)*	93	USA	BMD	≤2, FDA	5	2001–2005
Garza-Gonzalez et al. (2010)	*Acinetobacter baumannii*	550	Mexico	BMD	≤1 > 2, BSAC	3	07/2006–06/2007
Denys et al. (2013)	*Acinetobacter baumannii*	2900	USA	BMD	≤2, FDA	MIC50/90 = 0.5/<2 mg/L	2005–2011
Sader et al. (2014)	*Acinetobacter spp*	1257	USA	BMD	≤2, FDA	MIC50/90 = 0.5/2 mg/L	2006–2012
Garcia et al. (2009)	*Acinetobacter baumannii*	208	Chile	Agar Dilution method	≤2, FDA	20	10/2005–12/2006
Rizek et al. (2015)	*Acinetobacter baumannii (MDR)*	47	Brazil	BMD	≤2, FDA	0	NS
Ahmed et al. (2012)	*Acinetobacter baumannii complex (CR)*	232	Pretoria, South Africa	VITEK-2	≤0.25 ≥ 8	24	2/7/2010
Seifert et al. (2006)	*Acinetobacter baumannii*	215	Europe and USA	BMD	≤2, FDA	14.9	1990–2003
Insa et al. (2007)	*Acinetobacter baumannii*	142	Spain	E-test	≤2, FDA	12	1/2003–7/2006
Capone et al. (2008)	*Acinetobacter baumannii (MDR)*	80	Italy	BMD		27.5	1/2004–6/2005
Cattoir et al. (2014)	*Acinetobacter baumannii (MDR)*	1161	France	BMD		MIC 90 = 1 mg/L	2004–2012
Papaparaskevas et al. (2009)	*Acinetobacter spp (imipenem -resistant)*	187	Greece	BMD		MIC50/90 = 1/1	12/2006–6/2007
Spiliopoulou et al. (2014)	*Acinetobacter baumannii 92.1 % MDR)*	441	Greece	E-test	≤2, FDA	NS	1/2006–12/13
Ricciardi et al. (2009)	*Acinetobacter baumannii (MDR)*	50	Italy	E-test	≤2, FDA	50	1/2008–1/2009
Zarkotou et al. (2012)	*Acinetobacter baumannii (CR)*	56	Greece	BMD	≤2, FDA	14	2008–2011
Buccoliero et al. (2011)	*Acinetobacter baumannii*	81	Italy	VITEK-2	≤2, FDA	0	2011
Sader et al. (2005)	*Acinetobacter spp*	326	worldwide	BMD	≤2, FDA	5.5	2000–2004
Mendes et al. (2010)	*Acinetobacter spp*	5127	worldwide	BMD	≤2, FDA	3	2005–2009

NS not specified, *FDA* Food and Drug Administration, *EUCAST* European Committee on Antimicrobial Susceptibility Testing, *BSAC* British Society for Antimicrobial Chemotherapy, *MDR* multidrug resistant, *RR* resistance rate, *BMD* broth microdilution

As for specific regions worldwide, tigecycline non-susceptibility in the Middle-East countries ranges from 2 to 81 % (Guven et al. 2014; Navon-Venezia et al. 2007; Kulah et al. 2009; Dizbay et al. 2008; Baadani et al. 2013; Araj and Ibrahim 2008; Al-Sweih et al. 2011). The highest rates reported come from Israel (Navon-Venezia et al. 2007) and Turkey (Guven et al. 2014) with tigecycline resistance percentages of 66 % and 81 %, respectively. Navon-Venezia et al. have used the E-test methodology, which has been reported to give higher tigecycline MICs. Guven et al., reported increase in tigecycline resistance among MDR *A. baumannii* from 12.5 % in 2008 to 81.3 % in 2011 respectively (Guven et al. 2014).

In Asia, non-susceptibility rates ranged from 14.2 to 57.6 % (Behera et al. 2009; Taneja et al. 2011; Liao et al. 2008; Chang et al. 2012; Teng et al. 2014; Kim et al. 2010; Jiang et al. 2014; Van et al. 2014; Tan and Ng 2007; Liu et al. 2008). In India, two studies reported 14.2 % and 57.6 % non-susceptibility rates (Behera et al. 2009; Taneja et al. 2011). The higher rate was reported among MDR *A. baumannii* in a limited sample. In Taiwan, two studies report a rate of 19 % (Liu et al. 2008) and 29 % for tigecycline non-susceptible *A. baumannii* (Teng et al. 2014), while another Taiwanese study testing MDR *A. baumannii* isolates showed a rate of 45.5 % (Chang et al. 2012). In Asia and Western Pacific region, non-susceptibility rate was reported to be 0.2 % (Farrell et al. 2010)

In the Americas, tigecycline non-susceptibility was $\leq$5 % in North America (Scheetz et al. 2007; Garza-Gonzalez et al. 2010). Also, Denys et al., as part of the Tigecycline Evaluation and Surveillance Trial (T.E.S.T.) and Sader et al. in the USA reported $MIC_{50/90}$ values of 0.5/$\leq$2 mg/L (Denys et al. 2013; Sader et al. 2014). In South America, non-susceptibility varied between 0 and 20 % (Garcia et al. 2009; Rizek et al. 2015).

In South Africa, non-susceptibility was reported to be 24 % (Ahmed et al. 2012). In Europe, non-susceptibility ranged from 0 to 50 % (Zarkotou et al. 2012; Buccoliero et al. 2012; Capone et al. 2008; Ricciardi et al. 2009; Seifert et al. 2006; Insa et al. 2007). Spiliopoulou et al. reported an increase in tigecycline resistance from 25.5 % in 2010 to 66.5 % in 2013. MIC_{90} in two T.E.S.T. surveys conducted in Greece and France was estimated to be 1 mg/L (Papaparaskevas et al. 2010; Cattoir and Dowzicky 2014). Two worldwide studies estimate non-susceptibility rate of *A. baumannii* to tigecycline to be 5.5 % and 3 % respectively (Sader et al. 2005; Mendes et al. 2010).

3 Mechanisms of Tigecycline Resistance in *A. baumannii*

Resistance mechanisms to tigecycline among *A. baumannii* are still not fully elucidated. Nevertheless, efflux pumps seem to play a vital role. Three efflux pumps, AdeABC, AdeFGH and AdeIJK that are part of the resistance-nodulation division family (RND), up to now, have been associated with resistance to tigecycline in this species. MexXY and AcrAB that have been reported to be implicated in tigecycline resistance among *Enterobacteriaceae* and *P. aeruginosa*, also belong to the RND family. AdeABC, AdeFGH and AdeIJK pumps are thee-component systems consisting of a membrane fusion protein (MFP), an inner membrane transporter, and an outer membrane factor (OMF) (Peleg et al. 2007). This three component system allows crossing of both the inner and the outer membrane (Coyne et al. 2011), making them very effective. All three proteins in each pump are co-transcribed (Marchand et al. 2004; Coyne et al. 2010b; Damier-Piolle et al. 2008). Members of the RND family are proton antiporters, using the proton gradient to power efflux, exchanging one H+ ion for one drug molecule (Paulsen 2003).

The *ade*ABC operon is found in 80 % of *Acinetobacter* isolates (Coyne et al. 2010a), *ade*FGH in 90 % (Coyne et al. 2010b) and *ade*IJK is considered intrinsic to the species and is found in all *Acinetobacter* isolates (Damier-Piolle et al. 2008). AdeABC pump is controlled by a two-component system (AdeRS), namely a response regulator (AdeR) and a sensor kinase

(AdeS) (Marchand et al. 2004). AdeFGH is controlled by the LysR-type transcriptional regulator AdeL (Coyne et al. 2010b) and AdeIJK by the TetR transcriptional regulator AdeN (Rosenfeld et al. 2012).

Several compounds have been reported to be substrates for the AdeABC system, including aminoglycosides, tetracyclines, fluoroquinolones, trimethoprim, chloramphenicol (Bratu et al. 2008) as well as cefotaxime (Magnet et al. 2001). Overexpression of the AdeABC has been observed in tigecycline-resistant *A. baumannii* and was associated with increased MICs of tigecycline (Bratu et al. 2008; Peleg et al. 2007; Ruzin et al. 2010). On the other hand, two other studies (Yoon et al. 2013; Deng et al. 2014) found no correlation between tigecycline MICs and the levels of AdeABC expression, suggesting the presence of other mechanisms of tigecycline resistance. It should be noted that increased expression of the *adeB* gene was also found in tigecycline-susceptible strains, which could indicate the role of the AdeABC efflux pumps on other functions necessary for the pathogenesis of clinical strains of *A. baumannii*, such as colonization, infection and the persistence of organisms in the host (Rumbo et al. 2013).

Amino acid changes in the AdeRS system have also been implicated in AdeABC overexpression, but their actual contribution remains uncertain. In AdeS, point mutations Asp30Gly (Coyne et al. 2010b) in the sensor domain, Met62Ile (Hornsey et al. 2010), in clinical isolates Thr153Met in the histidine box in spontaneous mutants (Marchand et al. 2004) and Arg152Lys in clinical isolates (Yoon et al. 2013) downstream from the putative His-149 site with presumable loss of phosphorylation, have been described. In AdeR, Asp20Asn near the site of phosphorylation (Higgins et al. 2010), Pro116Leu in the helix of the receiver domain (Marchand et al. 2004) and Glu219Ala in the DNA binding domain (Yoon et al. 2013) have been reported. Lastly, polymorphisms Ala94Val (Hornsey et al. 2010; Rumbo et al. 2013), Gly186V, Phe214Leu in the AdeS and Ala136Val in the AdeR (Rumbo et al. 2013) have also been observed.

The mutation Ala94Val might have been erroneously considered as a functional mutation (Hornsey et al. 2010). Further studies in two *A. baumannii* isolates recovered by the same patient detected multiple mutations, raising the possibility of a mixed infection or re-infection, as it could not be determined whether they were evolved from one another during tigecycline treatment (Hornsey et al. 2011).

Concurrent point mutations Gly103Asp in AdeS and Ala91Val in AdeR located immediately upstream of the putative -10 promoter sequence of the *adeABC* operon, in a lab mutant obtained after tigecycline exposure have been reported (Hornsey et al. 2011). IS*Aba*-1 insertion in the AdeS has been proposed as a mechanism of resistance (Ruzin et al. 2007; Sun et al. 2012). Sun et al. demonstrated that the truncated AdeS was able to interact with AdeR and then enhance the adeABC expression (Sun et al. 2012).

Nevertheless, in some cases overexpression of the AdeABC system could not be associated with changes in the AdeRS system (Bratu et al. 2008; Peleg et al. 2007; Sun et al. 2010; Hornsey et al. 2010), implying alternative ways of control. BaeSR two component system has been shown to positive regulate the expression of *adeA* and *adeB* in both clinical isolates and laboratory induced tigecycline-resistant strains (Lin et al. 2014).

AdeIJK has also been implicated in tigecycline resistance (Damier-Piolle et al. 2008; Rosenfeld et al. 2012; Rumbo et al. 2013; Amin et al. 2013). Alterations detected in AdeN in mutants overexpressing the AdeIJK were deletion of cytosine 582 and a 394-bp deletion of the 3′ part of the AdeN (Rosenfeld et al. 2012). Polymorphisms of the AdeN reported are His111Pro, Ile112Phe, Pro16Lys (Rumbo et al. 2013).

Studies have shown that overexpression of AdeFGH is associated with tigecycline resistance (Coyne et al. 2010a). AdeL point mutations, Val139Gly, Thr319Lys, insertion at position 981 of a thymidine leading to 300- and 200- increase in *adeG* have been described (Coyne et al. 2010b). In contrast to this

observation, Amin et al. reported that AdeL transcriptional factor and the AdeFGH pump does not contribute to antimicrobial resistance since deletion of *adeL- adeFGH* operon had no impact on antimicrobial susceptibility in the clinical isolates studied, raising the question about the reliability of the method of selecting mutants via exposure to antibiotics and inserting resistance cassettes rather than generating marker less gene deletions (Amin et al. 2013).

Sun et al., noted that 11 tigecycline-resistant isolates showed no increase in adeA, 7/11 showed response to 1-(1-naphthyl)-piperazine (NMP), which is an efflux pump inhibitor and 4/11 showed no response to NMP, indicating that additional pumps or completely different mechanisms might contribute to tigecycline resistance (Sun et al. 2014). The involvement of a new RND pump together with *tetA* (39) has been suggested as a mechanism of tigecycline resistance (Rumbo et al. 2013). Other mechanisms have also been proposed for tigecycline resistance. *TetX1* gene, a new resistance mechanism to tigecycline reported previously in *Bacteroides fragilis* strains, was detected in 12/64 (18.8 %) tigecycline non-susceptible *A. baumannii* isolates (Deng et al. 2014). The TetX protein modifies first and second generation tetracyclines and requires NADPH, Mg^{+2} and O_2 for its activity (Moore et al. 2005). Decreased susceptibility to tigecycline has been mediated by a mutation in *trm* encoding SAM-dependent methyltransferase that play a role in epigenetic regulation and antibiotic resistance (Chen et al. 2014). A frameshift mutation in *plsC*, encoding 1-acyl-sn-glycerol-3-phosphate acyltransferase observed in a mutant after gradient exposure to tigecycline was proposed as a mechanism of tigecycline resistance, by influencing the membrane's permeability to tigecycline (Li et al. 2015).

Research focused on outer membrane proteins (OMPs) in *A. baumannii* has demonstrated that inactivation of AbuO, an outer membrane, homolog of TolC from Escherichia coli, that is regulated by the transcriptional regulator SoxR, conferred increased susceptibility to tigecycline in a lab mutant (Srinivasan et al. 2015).

4 Discussion

A. baumannii is considered as one of the most significant pathogens, particularly in the hospital setting (Boucher et al. 2009). Multidrug-resistant (MDR) *A. baumannii*, defined as resistant to three or more classes of antibiotics is of great concern, since often the only antimicrobial treatment choices remain colistin and tigecycline. Tigecycline resistance has been observed during therapy, but resistant isolates have also been recovered from patients without any previous tigecycline administration, probably partly due to AdeABC overexpression induced by other antibiotics that are also substrates for the pump. In addition, resistance to tigecycline against MDR *A. baumannii*, even before the drug was commercially available, has been reported (Navon-Venezia et al. 2007; Kulah et al. 2009; Dizbay et al. 2008). The development of resistance to any particular agent has often been shown to correlate with its overall use in the population (Stein and Babinchak 2013). It seems that tigecycline-resistant *A. baumannii* rates are increasing ever since it was approved by the FDA (2005) and the European Medicines Agency (2006) (Stein and Babinchak 2013) but also maybe partly due to indiscriminate or off-label use, i.e. suboptimal concentration of tigecycline in serum could promote tigecycline resistance, making this superbug even more promiscuous. Notably, neither the branding company nor the official institutions CLSI and EUCAST recommend the use of tigecycline against *A. baumanni* due to insufficient data. The wild type MIC distribution of tigecycline in *A. baumannii* ranges between 0.064 and 1 mg/L. It is evident that tigecycline most probably cannot offer a bacteriostatic effect in bacteraemia, where the achievable serum concentration of tigecycline at normal dosing is 0.62–0.72 mg/L, a value below the FDA breakpoint (Karageorgopoulos et al. 2008). The use of tigecycline in tissue infections, where tigecycline reaches higher concentrations might be more promising. Taken together, these observations suggest that caution should be given to unreasonable use of

tigecycline in poorly penetrated anatomic sites, in order to restrain the development of further resistance.

A major role in tigecycline resistance in *A. baumannii* is exerted by the RND-efflux pumps, though the mechanisms of resistance are more complicated and diverse than what has so far been described and need to be further elucidated.

Tigecycline was shown to exhibit good *in vitro* bacteriostatic activity against *A. baumannii*, including strains resistant to imipenem (Pachon-Ibanez et al. 2004). Additionally, tigecycline has shown considerable, though not consistent, antimicrobial activity against MDR, including carbapenem-resistant, *Acinetobacter* spp. (Karageorgopoulos et al. 2008). Uncertain clinical efficacy regardless of excellent *in vitro* activity of tigecycline (MIC < 2 mg/L) against MDR *A. baumannii* has been reported, suggesting poor correlation between clinical and microbiological outcome (Gordon and Wareham 2009). It has been reported that *A. baumannii* isolates with tigecycline MICs of >2 mg/L were associated with higher mortality rate and that pre-therapy MIC determination of tigecycline against *A. baumannii*, may predict clinical success (Anthony et al. 2008). Another study from Taiwan that compared the effectiveness of tigecycline- versus colistin-based therapy for the treatment of pneumonia caused by MDR *A. baumannii* revealed that the excess mortality rate in the tigecycline-based group observed compared to the colistin-based group was significant only among those patients with MIC >2 μg/mL but not for those with MIC ≤ 2 μg/mL (Chuang et al. 2014). In a systematic review and meta-analysis of the efficacy and safety of tigecycline, increased mortality, clinical failure and rate of septic shock development was observed with the use of tigecycline (Yahav et al. 2011). It has also been reported that when tigecycline therapy and non-tigecycline therapy was compared in terms of survival rate for the treatment of infections due to MDR *A. baumannii*, no significant difference was found between the two groups, although the rate of unfavourable outcome was significant lower in the tigecycline group (Lee et al. 2013). The FDA, in a drug safety communication recommended that health care professionals should reserve tigecycline for use in situations when alternative treatments are not suitable, based on an analysis showing increased risk of death when tigecycline was used compared to other antibacterial drugs (FDA 2013).

In the absence of established interpretative criteria for in vitro susceptibility testing, the non-susceptibility of tigecycline in *A. baumannii* cannot be accurately validated. Nevertheless, when tigecycline is intended to be used, it is important to confirm the *in vitro* susceptibility test using the recognized standard of broth microdilution (Bradford et al. 2005) in order to avoid any discrepancies. It seems that more light should be shed to the activity of the drug against *A. baumannii* so that official institutions could establish interpretative criteria for in vitro susceptibility testing.

In conclusion, it is evident that the status of tigecycline against *A. baumannii* remains obscure. On one hand, patients with life-threatening infections due to MDR *A. baumanni* isolates demand an effective confrontation, on the other hand approved indications of tigecycline are limited and its clinical effect against A. *baumanni* is uncertain. Tigecycline is still an attractive choice for *A. baumannii*, but further investigations are warranted so that treatment of MDR *A. baumannii* could be guided by validated data.

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In Vitro and *In Vivo* Biofilm Wound Models and Their Application

Gilles Brackman and Tom Coenye

Abstract

Chronic wounds are wounds which are detained in one or more phases of normal wound healing. It is estimated that 1–2 % of the population of developed countries will experience a chronic wound during their lifetime and this number is expected to increase given the growing world population, increase in age, body mass index and associated diseases such as diabetes and cardiovascular diseases. Although several factors contribute to wound healing, presence of bacterial biofilms significantly affects healing and success of wound treatment. This indicates that wound-care therapies should be directed towards targeting biofilms within chronic wounds. Despite this, the role of biofilms in chronic wound pathogenesis and the effect of wound-care therapies against biofilms within wounds are not well understood. In order to address these issues, appropriate biofilm models are necessary. To this end, several model systems mimicking the conditions observed in a biofilm infected chronic wound have been developed. In this review we present an overview of these different *in vitro* and *in vivo* biofilm wound model systems and discuss their advantages and disadvantages.

Keywords

Chronic wounds • Biofilms • *in vitro* wound biofilm models

1 Introduction

Chronic wounds are wounds which are detained in one or more phases of normal wound healing (Lazarus et al. 1994). Diabetic, arterial, venous and pressure ulcers constitute the majority of these wounds. Chronic wounds affect between two and seven million of patients annually with

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treatment costs rising up to several billions of dollars annually (Sen et al. 2009). It has been estimated that 1–2 % of the population of developed countries will experience a chronic wound during their lifetime and this number is expected to increase given the growing world population, increase in age, body mass index and associated diseases such as diabetes and cardiovascular diseases (Gottrup 2004). Although several factors contribute to wound healing, bacterial infections can significantly affect healing and success of wound treatment (Robson 1997; White and Cutting 2006; Wolcott et al. 2010b). The moist environment and the constant supply of nutrients within the wound represent the ideal environment for bacterial growth. These bacteria can come from different exogenous (e.g. soil and water) as well as endogenous (e.g. skin, saliva, urine, faeces) sources. However, the biodiversity is suggested to be relatively low and *Staphylococcus aureus* and *Pseudomonas aeruginosa* seem to predominate in chronic wounds (Bowler 1998; Fazli et al. 2009; Gjodsbol et al. 2006; Kirketerp-Moller et al. 2008; Rao and Lipsky 2007; Rhoads et al. 2012).

Increasing evidence suggest that these bacteria reside within biofilms in these wounds (Bjarnsholt et al. 2008; Burmølle et al. 2010; Church et al. 2006; James et al. 2008). Biofilms are sessile communities characterized by microbial cells that are irreversibly attached to a substratum and/or to each other and are embedded in a self-produced matrix of extracellular polymeric substances and exhibit an altered phenotype compared to planktonic cells (Costerton et al. 1999). Bacteria living in these biofilms are well protected against antimicrobial agents and host defenses and are for that reason extremely difficult to eradicate (Fux et al. 2003; Bjarnsholt et al. 2008). Recent studies have shown that the major reason for the failure of wound treatment and the shift from acute towards a chronic wound is the presence of bacterial biofilms within the wounds (Harrison-Balestra et al. 2003; Bjarnsholt et al. 2008; Davis et al. 2008; Kirketerp-Møller 2008; Kirketerp-Møller and Gottrup 2009). Only 6 % of acute wounds contained biofilms while this was between 60 and 80 % for chronic wounds (James et al. 2008). In addition, in a study of Dowd et al. (2009) only wounds without detectable biofilm showed signs of wound healing. This indicates that wound-care therapies should be directed towards targeting biofilms within chronic wounds. Despite this, the role of biofilms in chronic wound pathogenesis and the effect of wound-care therapies against these biofilms are not well understood. In order to address these issues, appropriate biofilm models are necessary. To this end, several model systems mimicking the conditions observed in a biofilm infected chronic wound have been developed. In this review we present an overview of these different *in vitro* and *in vivo* biofilm wound model systems and discuss their advantages and disadvantages.

2 Static *In Vitro* Wound Models

Different biofilm models have been used to evaluate the effect of antimicrobial agents on biofilms (see Coenye and Nelis 2010 for a general overview of biofilm model systems). These “general purpose models” can be used to evaluate the efficacy of wound care products or to evaluate biofilm formation of wound isolates. However, most of these *in vitro* models do not reflect the micro environmental conditions found in the wound bed. For this reason, several researchers have made specific adaptations to these general static biofilm models trying to better mimic wound-like environments in an easy-to-handle *in vitro* setting. For example, static biofilm models were developed in which biofilms were grown on agar, poloxamer gels or cellulose matrixes placed in petri-dishes (Clutterbuck et al. 2007; Percival et al. 2007; Hammond et al. 2011; Merritt et al. 2011; Kim and Izadjoo 2015) (Table 1). Although poloxamer gels are polysaccharides, bacterial cultures growing on this substrate mimic many of the properties of biofilm-grown bacteria. Similarly, the permeable nature of cellulose disks allows diffusion of nutrients to the bacteria on the disk, just as nutrients are supplied to biofilms in a wound. As such, both set-ups have been used to evaluate

Table 1 Overview of different static *in vitro* chronic wound models

Characteristics	Percival et al. (2007)	Sun et al. (2008)	Werthén et al. (2010)	Kostenko et al. (2010)	Hammond et al. (2011)	Kucera et al. (2014)
Designation	Poloxamer model	LBCW	Collagen wound model	MBEC wound model	Cellulose agar model	Artificial wound bed model
Use of a wound like surface	No	No/Yes	Yes	Yes	No	No
	(Poloxamer gel)	(plastic tip, silicone disk or host-derived matrix)	(Collagen matrix)	(Serum coated pegs)	(Cellulose disks)	(Plastic)
Use of a wound like medium	No	Yes	Yes	No	No	Yes
	(MH-agar)	(Bolton Broth, 50 % bovine plasma, 5 % freeze-thawed lacked horse-blood)	(SWF: 50 % fetal calf serum and 50 % physiological NaCl in 0.1 % Pepton (PW) or a 1:1 TSB-SWF solution)	(TSB)	(LB-agar)	(Bolton Broth, 1 % gelatine, 50 % porcine plasma, 5 % freeze-thawed porcine erythrocytes or Bolton broth +1 % gelatine +1.2 % agar)
Air-liquid interface	Yes	No	Yes	No	Yes	Yes
Flow present	No	No	No	No	No	No
Inoculum	10^5–10^6 CFU	10^4 CFU	10^4–10^5 CFU	10^7 CFU/ml	10^2–10^4 CFU	10^4 CFU
Incubation temp	25–35 °C	37 °C	35–37 °C	37 °C	37 °C	37 °C

the effect of silver containing dressings (Percival et al. 2007, 2011), antibiotic ointments and agents (Clutterbuck et al. 2007; Hammond et al. 2011; Miller et al. 2014) and garlic (Nidadavolu et al. 2012). In addition, Kostenko et al. (2010) evaluated the efficacy of silver containing dressings using an MBEC ("Minimal biofilm eradication concentration") device. This set-up allows a non-destructive transfer of the biofilms into fresh medium. Biofilms in this device grow on pegs attached to the lid of the device which were coated with serum. Although, most of these general batch culture models have the advantage of being simple and allowing high throughput screening in a cost-effective manner and although some adaptations have been made to better reflect a wound environment, none of them convincingly mimics the conditions observed in an *in vivo* wound.

2.1 Lubbock Chronic Wound Biofilm Model and Derived Models

The first chronic wound model that truly attempted to mimic wound like conditions was developed at the medical biofilm research institute in Lubbock (Texas, US) and was therefore named the "Lubbock chronic wound biofilm model (LCWB)" (Table 1) (Sun et al. 2008). This model allowed the rapid (24 h) cultivation of a robust multispecies biofilm in which *P. aeruginosa*, *S. aureus* and *Enterococcus faecalis* are present in roughly equal ratios. These bacteria were chosen since they are often isolated from and co-occur in chronic wounds (Sun et al. 2008; Gjodsbol et al. 2006). However, the LCWB allows growth of several Gram negative and Gram positive bacteria, aerobes as well

as anaerobes (DeLeon et al. 2014; Dalton et al. 2011). An inoculum of 10^4 cells was used to represent a normal microbial load of a wound prior to infection. Biofilms are grown in a medium consisting of a chopped meat-based medium (Bolton broth) with 50 % heparinized bovine plasma and 5 % freeze-thaw laked horse red blood cells. As such the medium presents the major host factors (e.g. damaged tissue, red blood cells and plasma) found in a typical wound bed. A major downside of this model is the fact that biofilms are formed using a plastic tip or silicone disks as a substrate, which does not reflect a wound-like surface (Sun et al. 2008; Brackman et al. 2011). However, it was recently shown that the medium coagulates into a jelly-like mass when a coagulase-positive bacterial species is used (such as *S. aureus*). *S. aureus* secretes staphylocoagulase which binds to prothrombin, forming a complex which converts soluble fibrinogen to insoluble fibrin. As such there is no need for using an artificial surface since a host-derived matrix is formed which can serve as a scaffold to which bacteria can adhere and form biofilms (DeLeon et al. 2014). Another encouraging aspect of this *in vitro* model is the morphological similarity that is being observed, both with the naked eye as well as on electron micrographs, between biofilms grown in the model and biofilms on actual chronic wounds. As such, this model was shown to be a realistic *in vitro* model which is easy to handle and allows rapid growth and maturation of a multispecies biofilm in a cost effective manner. For this reason, the LCWB has been used extensively to study interspecies interactions (Dalton et al. 2011; DeLeon et al. 2014) and to assess the effect of antibiofilm compounds, antimicrobial agents, hydrogels, functionalized gauzes and dressings against both single species biofilms and polymicrobial communities (Garcia-Fernandez et al. 2013; Luna-straffon et al. 2014; Douglas et al. 2014; Sun et al. 2009; Dowd et al. 2009; Brackman et al. 2011).

Since the first publication, several research groups have made adaptations to the LCBW model to address specific needs. The evaluation of the effect is typically based on quantification of the number of biofilm cells by plating or by using quantitative qPCR methods, making it less suitable for screening large amounts of compounds. Recently, the LCWB was modified for high throughput testing to address this need (Brackman et al. 2013). A good correlation was observed between the fluorescence from a fluorescent *S. aureus* strain and the number of biofilm cells present after treatment (Brackman et al. 2013).

The LCWB model is often used to obtain polymicrobial wound-like biofilms which are then transplanted into other *in vitro* and/or *in vivo* models of skin infection. For example, Dalton et al. (2011) successfully transplanted a biofilm cultured in the LCWB model into a murine skin wound to induce *in vivo* formation of wound biofilms. In addition, Kucera et al. (2014) developed an artificial wound bed model for assessment of solid antimicrobial dressings based on the LCBW model. In brief, the biofilm was pre-cultured using the LCBW set-up with some modifications and amendments. These included the addition of gelatin to the wound medium and the use of porcine plasma and freeze-thaw lacked porcine erythrocytes instead of bovine plasma and horse blood. This pre-cultured biofilm was then transferred onto an artificial wound bed. This artificial wound bed consists of a two-layer nutrient medium composed of Bolton Broth supplemented with 1 % gelatin (w/v) and 1.2 % agar (w/v). The use of the artificial wound bed in the model enables to mimic the situation in chronic infected wounds where the biofilm is only in partial contact with the wound dressing. The modified set-up also incorporates an air-liquid interface feature which is usually present in wound biofilms.

2.2 Collagen-Based *In Vitro* Wound Models

In *in vitro* models, biofilms are often formed on solid, artificial surfaces. This makes it difficult to correlate the *in vitro* results with *in vivo* observations, since the full contribution of the surface to biofilm formation and biofilm

persistence is often unknown. In addition, bacteria in wounds are often not attached to well-defined solid surfaces, but instead reside in the wound bed. For this reason, a model system in which sessile bacteria are aggregated in the absence of a solid surface would mimic the conditions in the wound more closely. To address this issue, Werthén et al. (2010) developed an *in vitro* wound model in which biofilms can develop in the presence of simulated wound fluid (containing 50 % fetal calf serum and 50 % physiological saline in 0.1 % peptone) and a matrix of polymerized rat-tail collagen type I but in the absence of a solid surface (Table 1). Both *P. aeruginosa* and *S. aureus* formed aggregates, surrounded by self-produced polysaccharide matrix within the collagen matrix (Werthén et al. 2010). In addition, biofilms formed in this model were structurally similar to biofilms observed *in vivo*, suggesting the presence of a "wound-like" environment (Werthén et al. 2010). The deep penetration of *P. aeruginosa* biofilms and the more surface-oriented biofilms of *S. aureus* observed in this model resembled other *ex vivo* observations (Kirketerp-Moller et al. 2008). For this reason, this model was used to better predict the *in vivo* antimicrobial activity of antibiotics and silver-containing wound-dressings in several studies (Brackman et al. 2011; Hakonen et al. 2014).

3 *In Vitro* Chronic Wound Models with Liquid Flow

Although the above mentioned biofilm models aim to mimic *in vivo* wound-like environments, all of them are based on closed and therefore accumulative batch culture systems. For this reason, some argue that it is unlikely that they will fully represent the true dynamic state of the wound environment. To address this issue several *in vitro* wound models were developed in which a fluid flow is present and/or in which the biofilm is exposed to shear stress (Thorn and Greenman 2009; Lipp et al. 2010; Hill et al. 2010) (Table 2). The *in vitro* flat-bed perfusion model (Thorn and Greenman 2009), developed based on previously described static models (Greenman et al. 2006; Thorn et al. 2007) addresses this issue. This model consists of autoclavable removable cassettes containing microscope slides on which 1 cm^2 cellulose matrices are placed. A hyperdermic needle, linked to a peristaltic pump was used to perfuse growth medium through the removable cassettes. The medium consists of 0.1 % heat-inactivated foetal calf serum (FCS) or 2 % FCS + 0.1 % glucose in phosphate buffered saline depending on whether *P. aeruginosa* or *S. aureus* was used, respectively (Thorn and Greenman 2009). This model can be used to determine the antimicrobial kill kinetic profile of topically applied treatments (Thorn et al. 2009). In addition, a bioluminescent target organism was integrated into the model and shows the feasibility of using light production for real-time monitoring of antimicrobial efficacy (Thorn and Greenman 2009).

Similarly, Lipp et al. (2010) used a colony drip-flow reactor (C/DFR) model to grow *P. aeruginosa* and *S. aureus* biofilms under wound-like conditions. This model was based on characteristics of both the colony biofilm model (Anderl et al. 2000) and the drip-flow reactor (DFR) model (Buckingham-Meyer et al. 2007). In the C/DFR, biofilms are grown on semipermeable membranes which are placed on microscope slides in a DFR apparatus. These membranes are inoculated with approximately 10^4 CFU of a single species (*P. aeruginosa* or *S. aureus*), left for 30 min to allow drying of the inoculum after which medium (10 % TSB) was pumped through the system (5 ml/h/channel) and biofilms were allowed to form for up to 72 h at room temperature (Lipp et al. 2010). Although initially single species biofilms were grown, growth of a polymicrobial biofilm consisting of bacteria with variable oxygen requirements is possible in this model (Woods et al. 2012). Interesting is the fact that growth of a strict anaerobe (*C. perfringens*) occurred in a polymicrobial biofilm with *P. aeruginosa* and *S. aureus* in the C/DFR, without establishing an artificial anaerobic environment (Woods et al. 2012). As such this model was used to evaluate the effect of antimicrobial agents (Agostinho et al. 2011) and

Table 2 Overview of different dynamic *in vitro* chronic wound models

Characteristics	Thorn and Greenman (2009)	Lipp et al. (2010)	Hill et al. (2010)	Ngo et al. (2012)	Terry and Neethirajan (2014)
Designation	Flat-bed perfusion model	C/DFR	CDFF	CDC-TNP model	Microfluidic wound model
Use of a wound like surface	No	No	Unclear	No	Yes
	(Cellulose matrix)	(Absorbant pad)	(not disclosed)	(Borosilicate or Teflon)	(Collagen)
Use of a wound like medium	Yes	No	No	No	No
	(Foetal calf serum (FCS) or 2 % FCS + 0.1 % glucose in PBS)	(10 % TSB)	(TSB or BM)	(TSB or 10 % TSB)	(TSB + 1 % glucose)
Air-liquid interface	Yes	Yes	Partly[a]	Partly[a]	Partly[a]
Flow present	Yes	Yes	Yes	Yes	Yes
	(1 ml/h)	(5 ml/h)	(30 ml/h)	(11.7 ml/min–40 ml/h)	(100–200 µl/h)
Inoculum	10^5 CFU	10^4 CFU	ND	ND	ND
Incubation temp	37 °C	RT (21.5 °C)	37 °C	30–37 °C	35 °C

[a]An air-liquid interface can be present at different stages (e.g. attachment step, biofilm formation step, evaluation of antibacterial therapies), but not during the entire experiment
ND specific number of cells is not disclosed

wound dressings (Lipp et al. 2010) against mono- and three-species biofilms (Woods et al. 2012).

Recently two different models were developed in which biofilms were first grown in a flow-displacement model and then transferred to an adapted novel *in vitro* wound-like set-up (Ngo et al. 2012; Hill et al. 2010; Malic et al. 2011). These two models are the constant depth film fermenter (CDFF) and the Centers for Disease Control (CDC) biofilm reactor. Both models allow the generation of identical, multiple biofilms simultaneously and allow to vary key parameters including nutrient source, temperature, oxygen availability and substrata (Pratten and Wilson 1999). The reproducibility of identical biofilms, the possibility to image biofilms in three-dimensions and in real-time makes these models interesting starting points to make biofilms which can be implemented in other models.

The CDFF consists of a glass chamber housing a rotating stainless steel disc in which a total of 15 sampling pans, each containing five plugs, are placed. The disc is placed at a set depth and rotates while a scraper plate aids in the distribution of medium across the plugs and maintains a constant depth of the biofilm by removing biofilm cells growing higher. Similarly, the CDC reactor consists of a glass vessel with eight removable polypropylene rods, each holding three removable coupons on which biofilms can form (Donlan et al. 2004). These are oriented in such a way that the coupon is perpendicular to the rotating baffle (Buckingham-Meyer et al. 2007). The glass chamber of both models contains both entry and exit ports allowing a continuous flow of fresh medium through the system. Hill et al. (2010) used a constant depth film fermentor (CDFF) to form multispecies biofilms consisting of wound isolates. In brief, biofilms were grown at 37 °C on plug inserts into the CDFF placed at a 400 µm depth. BM (Hill et al. 2010) or BHI (Malic et al. 2011) medium was pumped through the system at a rate of 30 ml/h. After biofilm formation, biofilms were transferred to a moistened dressing in a sterile petridish (Hill et al. 2010). This set-up has been used to evaluate the effect of different antibiotics, commercial dressings and anti-biofilm compounds (Hill et al. 2010). In addition,

this model was further used to evaluate co-aggregation, synergy and antagonism between bacteria isolated from different types of wounds (Hill et al. 2010; Malic et al. 2011). Similarly, a CDC biofilm reactor was used to form single species biofilms which are then placed in an *in vitro* wound model (Ngo et al. 2012; Valente et al. 2014). In brief, biofilms were grown in a CDC biofilm reactor on borosilicate coupons at 30 °C using Trypton soy broth which was supplied at a rate of 11.7 ml/min. After biofilm formation, coupons were taken out of the CDC and embedded into an agar base representing a low nutrient and moist organic wound surface. A major difference with the CDFF set-up was that a constant flow of 1 % TSB at 40 ml/h was provided across the agar surface by an intravenous infusion (Ngo et al. 2012). This model is mainly used to evaluate the effect of negative pressure by itself or in combination with silver impregnated foam dressings on wound biofilms (Ngo et al. 2012; Valente et al. 2014).

4 Microfluidic Wound Models

A major downside of most of the above mentioned methods is the need for relatively large amounts of test-compounds when evaluating their efficacy in these models. Microfluidic-based wound models can overcome this drawback (Zhang et al. 2013). Microfluidic technology is a relatively new field that is already applied to study biofilm growth in a confined space (e.g. mimicking biofilm growth in a blood vessel) (Sato et al. 2014), to study antimicrobial resistance in biofilms by creating dynamic concentration gradients and/or to study spatial and temporal growth of micro-organisms as well as motility and chemotaxis in biofilms (Kim et al. 2012; Halder et al. 2013). Although differences between microfluidic devices exist, the channels are typically 50–500 μm wide, 30–500 μm deep and 1–40 mm in length. In addition, flow rates are usually low (0.1–50 μl/min) (Coenye and Nelis 2010). Recently, a "microfluidic wound model" was described which is easy to use, relatively cheap and small (Terry and Neethirajan 2014) (Table 2). In order to better mimic wound like surfaces, the channels were coated with rat tail collagen type I before bacteria were pumped through the system (Terry and Neethirajan 2014; Chen et al. 2014).

Although microfluidic wound models have several advantages compared to other models (e.g. use of a flow while only small amounts of test product are needed) there is still room for improvement on different other levels (e.g. use of more relevant media, surfaces and mixed biofilms). In this view it is interesting to note that microfluidic co-culture models are being developed in which biofilms can develop in the presence of an epithelial cell monolayer (Kim et al. 2010a, b; Zhang et al. 2013). Recently, Zhang et al. (2013) developed a microfluidic wound-scratch model system to investigate cell migration and proliferation. Although this model was not published in the context of infected wound biofilms, it displays the possibility of upgrading existing models to better emulate the conditions observed in an infected *in vivo* chronic wound.

5 Issues with the *In Vitro* Wound Models

Although all of the above mentioned *in vitro* models address specific aspects of wound biofilms, they all are prone to limitations (Tables 1 and 2). First of all, although some models display flexibility in the use of different bacterial species and/or mixed biofilm communities, most of the *in vitro* wound models only rely on the use of a single bacterial species. As such it is unclear whether these models would allow the incorporation of a biofilm consisting of different bacterial species. Dominant single species biofilm aggregates of *S. aureus* and *P. aeruginosa* are observed in infected chronic wounds and the outcome of wound healing can be correlated with the presence of a specific species. However, infected chronic wounds are often polymicrobial in nature, despite the fact that bacterial diversity is generally low (Robson 1997; Rao and Lipsky 2007; Colsky et al. 1998;

Gjodsbol et al. 2006; Fazli et al. 2009; Rhoads et al. 2012). For this reason, increasing the complexity of the model by adding multiple species could make the model system more relevant.

A second issue is the temperature used. Most of these biofilms are formed and maintained at 37 °C which reflects core body temperature. However, although skin temperature can be different due to variability between persons and body location, temperature of trauma wounds and wound bed temperature of chronic leg ulcers ranges between 25–37 °C and 24–26 °C, respectively (Fierheller and Sibbald 2010; Romanelli et al. 2002). This temperature is significantly lower than what is often used in the different models, which would indicate that conducting experiments at lower temperatures would better reflect the chronic wound bed temperature.

A wide range of different inocula are also being used in these models. These inocula range between 10^2 and 10^8 CFU. It is generally accepted that infected chronic wounds contain more than 10^5 bacteria per gram of tissue (Robson 1997; Bowler 2003). Although it is highly questionable that high bioburden levels are present at the start of infection under proper standard care conditions, models applying these higher inocula might be representative for heavily infected wounds or wounds inflicted under conditions were proper wound-care is not directly possible. In addition, lower inocula can be used for investigating biofilm development from the start of an infection. As such the inoculum used, should depend on the question that needs to be answered and it should be clear whether different inocula can be used in the different model systems.

Thirdly, the surface and media used in some models often do not reflect the nutritional conditions which bacteria would find in wound beds. Surfaces such as glass, silicone and plastics do not resemble the surfaces on which biofilms are formed in real wounds. In addition, although some artificial surfaces (such as poloxamer gels and cellulose disks) do possess some wound-like features, it remains questionable whether these would evoke similar responses in bacterial gene expression, biofilm formation and resistance to therapy as to biofilms grown on biotic surfaces. As such, most of these *in vitro* models do not take into account the role that dermal substrates can play on bacterial attachment, nutrition, biofilm shape and resistance and for this reason these models could be adapted at the level of the surfaces used in order to better mimic wound like conditions. Similarly, general media such as TSB or LB support the growth of a wide variety of microorganisms, but they do not contain many of the components which are present in wound exudates. Specific media such as the simulated wound fluid (Werthén et al. 2010) or media containing plasma, serum, blood cells and/or heparin likely better reflect nutritional conditions observed in wounds. However, to date there is no standardized nutrient medium to replicate wound exudates under *in vitro* conditions and the composition of wound fluid and wound exudates can be highly variable depending individual, type of wounds and wound healing stadium (Trengove et al. 1996, 1999; Cutting 2003; Eming et al. 2010). It thus remains difficult to really define which media would reflect wound conditions best.

Finally, as crucial is the expected geometry of how nutrients are applied to the wound biofilm. Although this might vary depending on the wound type and amount of exudate produced, nutrients generally originate from the host tissue at the bottom of the biofilm, while oxygen is usually supplied from the top of the biofilm at the air-liquid/surface interface. In addition, the physical aspect of a low fluid shear might be important in specific wound types. Although most of the *in vitro* wound model systems take into account one or more of these aspects in order to mimic *in vivo* wounds, none of them take into account all these aspects (Tables 1 and 2).

6 Cell-Based Wound Models

Implementing skin as a substrate for attachment and as the primary source of nutrition for microbial biofilm cells would allow the formation of biofilms under conditions which would more closely resemble the *in vivo* situation. For this

reason, several more advanced cell-based wound models were developed in which porcine skin explants (Yang et al. 2013; Phillips et al. 2013; Wolcott et al. 2010a), two-dimensional cell monolayers or three-dimensional tissue-engineered human skin equivalents (TE-HSE) (Haisma et al. 2013; Charles et al. 2009) were used as a substrate for biofilm development.

Given the fact that pig skin and human skin have striking similarities in structure (Summerfield et al. 2014), cell-based wound models using porcine skin explants have been used to study molecular characteristics of biofilms attaching to skin (Yang et al. 2013), assess the efficacy of antimicrobial agents and antimicrobial wound care dressings against *P. aeruginosa* and *S. aureus* biofilms and assess the effect of negative pressure wound therapy with instillation of antimicrobial solutions against *P. aeruginosa* biofilms (Phillips et al. 2010, 2013). A main disadvantage is that significant differences still exist between human and animal skin at the level of immunological responses (Summerfield et al. 2014). Despite this, human explants have rarely been used since it would be difficult to standardize and reproduce results obtained in such models. The development of reconstituted human tissue models using two-dimensional cell monolayers or three-dimensional tissue-engineered human skin equivalents would overcome this issue. Although monolayer cultured cells are often used, such studies do not accurately reflect the behavior, pathophysiology, or microenvironment of skin *in vivo* (Welss et al. 2004). Cells in monolayer culture are in isolation and for this reason do not take into account that bacteria invade and interact with different cell types in a complex three-dimensional solid structure. For this reason, three-dimensional systems would better mimic *in vivo* infections. Tissue-engineered human skin equivalents (HSE) are three-dimensional systems that mimic the native skin to a high degree (Welss et al. 2004). Although different HSE are described in literature, they are typically generated by culturing primary keratinocytes and dermal fibroblasts at the air-liquid interface of cell-free matrices (e.g. filters, collagen gels or decellularized dermal scaffolds such as de-epidermized dermis). The cells will proliferate, migrate and differentiate during peridermal development resulting in skin equivalents that usually contain all layers of the native epidermis and/ or dermis (El Ghalbzouri et al. 2004, 2008; Charles et al. 2009; Welss et al. 2004; Torkian et al. 2004). In addition, several HSE are commercially available. Epiderm-FT (MatTek, MA, US) is a multilayered highly differentiated skin model consisting of human-derived keratinocytes and fibroblasts in cell culture inserts. Apligraf is a tissue engineered skin equivalent which consists of a lower dermal layer (collagen and human fibroblasts) and an upper epidermal layer (human keratinocytes which can differentiate). In addition, reconstructed human epidermis (RHE, Skinethic, Lyon, France) consists of normal human keratinocytes cultured on an inert polycarbonate filter at the air-liquid interface, in a chemically defined medium. The HSE is typically wounded using a biopsy punch or a device heated or cooled with boiling water or liquid nitrogen, respectively, prior to infection (El Ghalbzouri et al. 2004; Haisma et al. 2013; Shepherd et al. 2009). Others have demonstrated that bacteria can colonize HSE and trigger the expression of pro-inflammatory cytokines/chemokines by the underlying cells (Holland et al. 2008, 2009; De Breij et al. 2012; Haisma et al. 2013; Kirker et al. 2009, 2012; Charles et al. 2009). In addition, HSE wound models were used to assess the antimicrobial activity of different agents and plasma against bacterial biofilms under wound like conditions (Haisma et al. 2013; Shepherd et al. 2009; Brackman et al. 2011).

Recently, Bellas et al. (2012) developed a full-thickness skin equivalent which included epidermis, dermis, and hypodermis. This model would serve as a more physiological relevant system that would likely sustain physiological function for more extended time periods in ways that would permit both acute, short-term, and chronic, long-term studies of skin development and pathogenesis. In addition, the morphology and organization of the tri-layer skin model

would allow secretion of appropriate levels of cytokines and mimic the full spectrum of biological functions of skin. The cell-based models have the advantage that they are histologically similar to human skin and thereby provide a controlled environment similar to the one encountered in *in vivo* wounds. However, unlike human skin, these usually do not contain Langerhans' cells, macrophages, lymphocytes or other structures such as blood cells, hair follicles or sweat glands.

7 *In Vivo* Wound Model Systems

To address the above mentioned issues, several *in vivo* wound models were developed, each with their own strengths and weaknesses (Seth et al. 2012). These animal models are needed since it is virtually impossible to study the development of chronic wound in humans. This is due to ethical concerns, but also due to the fact that the chronic wound is often already present when patients arrive in the clinic. In addition, when these wounds are investigated, this will only be observational thereby lacking the experimental and causative data necessary to fully investigate the role of biofilms and interplay with therapeutically agents (Seth et al. 2012).

One of the first studied *in vivo* models of wound infections relied on the use of *Drosophila melanogaster* (reviewed by Apidianakis and Rahme 2009). A wound infection in the cuticular epithelium and underlying muscle is established in this model by using a thoracic or abdominal pin prick which was dipped in a bacterial suspension. As such, this model was used to study host responses to wound infection by different microbes. Despite being often used, the translation of results obtained in an invertebrate pin-prick wound system to what could be expected in human wounds is questionable. For this reason, mostly vertebrate animals such as mice, rats, pigs and rabbits are used in *in vivo* wound model systems (Table 3). Next to the type and breed of animal used, these models mainly differ in the mechanisms by which wounds are inflicted, how wounds (and infection) is being maintained during the experiment, on the inoculum size and whether or not different bacterial species were shown to be capable of infecting the host under the given circumstances.

Akiyama et al. (1996) described biofilm formation of *S. aureus* in incisional wounds of mice and this model was later on used to evaluate topical treatment on biofilm susceptibility (Akiyama et al. 2002). Similarly, Rumbaugh et al. (1999) and later on Rashid et al. (2000) examined the role of different genes (including quorum sensing genes) on *P. aeruginosa* virulence in a burn wound mouse infection model. However, the effect of biofilm infection on the global wound healing process or host responses was not assessed. Similarly, several other murine infection models are published in which wounds are caused by thermal injury (Trøstrup et al. 2013; Nichols et al. 2013). Although these models can be useful to study burn wound infections, they do not always represent conditions found in chronic wounds which not originated from burns. For this reason several other models have focused on inflicting wounds by other manners such as biopsy punch (Thompson et al. 2014; Schierle et al. 2009; Zhao et al. 2010; Petreaca et al. 2012; Gurjala et al. 2011), surgical incision (Ermolaeva et al. 2011; Asada et al. 2012; Watters et al. 2014) or by means of sanding (Roche et al. 2012a, b) or pressure (Nakagami et al. 2008). Besides inflicting a wound, maintaining a biofilm infection within these models for a certain amount of time remains challenging. For this reason several models rely on specific preconditioned animals (e.g. mutant breeds or induction of specific pathogenesis such as diabetes), the pre-formation of the biofilm under *in vitro* conditions before the biofilm is applied to the wound bed and/or placement of dressing materials to maintain a moist environment (Table 3). Most of the rodent models also ignore the fact that contracture should be minimized in these models. By minimizing contractures, e.g. by placement of silicone rings around the wound bed, wounds are allowed to heal by new tissue ingrowth, more akin to human wounds, as opposed to myofibroblast-mediated

Table 3 Overview of different *in vivo* chronic wound models

Animals	Wound type	Wound location	Wound maintenance	inoculum	Single or mixed species biofilm	Reference
Murine models						
Ddy mice	Cut wounds	Back	–[a]	3.6×10^6 CFU/ml	Single (*S. aureus*)	Akiyama et al. (1996), (2002)
Swiss Webster mice	Thermal injury (90 °C water) (15 % t.b.i.)	Back	–[a]	10^2 CFU	Single (*P. aeruginosa*)	Rumbaugh et al. (1999), Rashid et al. (2000)
Swiss Webster mice	Surgical excision wound (1.5 cm^2)	Dorsal	Opsite dressing	10^4 CFU	Single (*P. aeruginosa* and *S. aureus*)	Watters et al. (2014), Turner et al. (2014)
						Wolcott et al. (2010a), Gawande et al. (2014)
BalB/c mice	Thermal injury (hot air) (6 % t.b.i.)	Back	Seaweed alginate beads	10^7 CFU/ml	Single (*P. aeruginosa*)	Trøstrup et al. (2013))
C57BL/6 J mice	Thermal injury (10 % t.b.i.) and abrasion injury	Dorsal	Abrasion injury prior to infection	10^6 CFU	Single (*P. aeruginosa*)	Nichols et al. 2013
Adult male C57Bl6/J mice	Excisional punch wounds	Back	Silicone rings and covered with Tegaderm	Pre-formed biofilm[b]	Single (*S. aureus* and *S. epidermidis*)	Schierle et al. (2009)
Male SWR/J and male TH mice	Full-thickness dermal wounds (ND)	Dorsal	Silicon splints and tegaderm dressing	10^6 CFU	Single (*S. aureus*)	Nguyen et al. (2013)
Db/db mice	Full-thickness punch wounds	Dorsal	Dressing occlusion	Pre-formed biofilm[b]	Single (*P. aeruginosa*)	Zhao et al. (2010)
BALB/c	Biopsy punch	Dorsal	Tegaderm dressing	5×10^4 CFU	Single (*A. baumannii*)	Thompson et al. (2014)
BALB/c mice	Full thickness wound (ND)	Back	Gauze patch	5×10^7 CFU	Single (*S. aureus*)	Simonetti et al. 2008
LIGHT−/− mice	Biopsy punch	Dorsal	tegaderm	10^8 CFU/ml	Single (*S. epidermidis*)	Petreaca et al. (2012), Dhall et al. (2014)
Mice (type not disclosed)	Wounded by sanding	Back	Moistened bandage	2×10^7 CFU	Single (*S. aureus*)	Roche et al. (2012a)
Adult male Sprague Dawley rats	Surgical incision	Nape (back of the neck)	Cotton pellets	10^8 CFU/ml	Mixed (*P. aeruginosa* and *S. aureus*)	Ermolaeva et al. (2011)

(continued)

Table 3 (continued)

Animals	Wound type	Wound location	Wound maintenance	inoculum	Single or mixed species biofilm	Reference
Adult male Sprague Dawley rats	Scissors incision	Flank region	Tegaderm dressing	2 × 10^9 CFU	Single (*P. aeruginosa*)	Asada et al. (2012)
male Wister rats	Pressure-related ischemic wounds	lateroabdominal and dorsal regions	–[a]	10^5 CFU	Single (*P. aeruginosa*)	Nakagami et al. (2008)
Pig models						
Commercially raised, specific pathogen-free, female Yorkshire-cross pigs	Full thickness trephine (2 cm) wounds	Back	Gauze pad and tegaderm dressing	10^7–10^8 CFU	Single (*S. aureus*)	Roche et al. (2012b)
Young, female, specific pathogen-free pigs	Wounded using a modified electrokeratome set	Back and side	polyurethane film dressing (tegaderm) and self-adherent bandages	10^6–10^7 CFU/mL	Single and mixed (*P. aeruginosa* and *S. aureus*)	Davis et al. (2001, 2007, 2008), Pechter et al. (2012), Pastar et al. (2013), Nusbaum et al. (2012)
Rabbit models						
New Zealand white rabbits	Full-thickness dermal punch wounds	Ventral side of each ear	Tegaderm dressing	10^6 CFU	Single and mixed (*K. pneumoniae*, *P. aeruginosa* and *S. aureus*)	Gurjala et al. (2011), Seth et al. (2012), Chen et al. (2014), Leung et al. 2014

[a]No specific wound-maintenance strategy mentioned
[b]Pre-formed biofilm. Specific inoculum number not disclosed
ND specific mechanism not disclosed

contraction of the loose rodent skin (Schierle et al. 2009; Nguyen et al. 2013). Additionally, only a limited amount of models study the infection for a longer period of time (Thompson et al. 2014; Roy et al. 2014). Although the use of mice and rats have some advantages over the use of larger animals such as pigs (e.g. ease-of-use, space-limitations, economical and ethical concerns), pigs are preferred for wound healing studies due to higher similarities between porcine and human skin and due to the scale at which wounds can be introduced (Sullivan et al. 2001; Summerfield et al. 2014). In addition, with respect to the translational value, the use of pigs as preclinical model for wound studies is recommended (Gordillo et al. 2013). Recently, an *in vivo* biofilm wound infection model was developed in rabbits (Gurjala et al. 2011). This model was based on the rabbit dermal ulcer model, which is an FDA-recognized model of wound healing which has been used for over two decades (Mustoe et al. 1991; Chen et al. 1999; Said et al. 2005; Mogford et al. 2009). In this model, full-thickness, circular punch-wounds are made in the ears of New Zealand White rabbits down to cartilage, affording a number of important advantages. For example, in contrast to partial-thickness wounds, this removal of dermis more closely models the dermal-loss seen in human chronic wounds. Additionally, the majority of human wounds heal through epithelialization and granulation, in contrast to the contracture-based healing seen in mice (Schierle et al. 2009). The underlying cartilage of the rabbit ear serves as a natural splint, preventing healing by contracture, and thus allowing for accurate quantification of epithelial and granulation tissue formation from the periphery of the wound. Moreover, multiple identical wounds can be made in one animal with contralateral controls, creating a standardized and high-throughput wound model. In contrast to other published models where pre-formed *in vitro* biofilm is directly applied to wounds, these wounds are inoculated with planktonic, free-floating bacteria which more closely represents the seeding mechanism of human chronic wounds, with the wound bed itself playing a critical role in the transformation of bacteria into the biofilm state (Schultz et al. 2004; Cierny and DiPasquale 2006). Although different *in vivo* models exist, the clinical relevance of these models is still being argued (Seth et al. 2012). These aspects should be addressed in the future.

8 Concluding Remarks

Investigating wound infections and development of novel therapeutic agents targeting these types of infections require the existence of appropriate models. As discussed in this review, several *in vitro* and *in vivo* wound model systems have been described, each with their specific strengths and weaknesses and addressing different aspects of wound biofilms. As such, researchers should select a model by measuring out these differences against the questions that they are hoping to answer using these models. However, due to the complexity of wound healing, extrapolation of results from *in vitro* biofilm studies to the clinic will always remain challenging. Only animal models can take into account factors such as interplay of immune reponses and wound bed components. In addition, *in vivo* animal models are necessary, since it is virtually impossible to study the development of chronic wound in humans. For this reason, there is a wide consensus that there is a high need for not only conducting these experiments, but also for a further development and improvement of the existing models both *in vitro* as well as *in vivo*. These modifications, including the introduction of polymicrobial biofilms, more relevant media and surfaces, would possibly lead to models which are truly capable of evaluating therapies under *in vitro* and *in vivo* settings. In addition, better models would eventually lead to studies on biochemical pathways (e.g. by use of mutants), host response to infection and on the interplay between different therapeutically agents and the biofilms which would better reflect reality. This would ultimately improve our understanding of why chronic wounds develop and why they are being maintained and altogether these insights

could possibly lead to better therapies addressing the issue of chronic wound infections in the clinic in the future.

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The *Lactobacillus plantarum* Eno A1 Enolase Is Involved in Immunostimulation of Caco-2 Cells and in Biofilm Development

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Abstract

The role of probiotics in prevention and treatment of a variety of diseases is now well assessed. The presence of adhesive molecules on the cell surface of probiotics has been related to the ability to confer health benefit to the host. We have previously shown that the enolase EnoA1 of *Lactobacillus plantarum*, one of the most predominant species in the gut microbiota of healthy individuals, is cell surface-expressed and is involved in binding with human fibronectin and plasminogen. By means of comparative analysis between *L. plantarum* LM3 (wild type) and its isogenic LM3-CC1 (*ΔenoA1*) mutant strain, here we show that EnoA1 affects the ability of this bacterium to modulate immune response as determined by analysis of expression of immune system molecules in Caco-2 cells. Indeed, we observed induction of TLR2 expression in cells exposed to *L. plantarum* LM3, while no induction was detectable in cells exposed to LM3-CC1. This difference was much less consistent when expression of TLR4 was determined in cells exposed to the two strains. Pro-inflammatory (IL-6) and anti-inflammatory cytokines (IL-10, TGF-β), and the antimicrobial peptide HBD-2 were induced in Caco-2 cells exposed to *L. plantarum* LM3, while lower levels of induction were detected in cells exposed to LM3-CC1. We also analyzed the ability to develop biofilm of the two strains, and observed a decrease of about 65 % in the development of mature biofilm in LM3-CC1 compared to the wild type.

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Keywords
L. plantarum LM3 • Adhesins • Immunomodulation • Biofilm

1 Introduction

The intestinal epithelium, once considered simply a physical barrier, is now considered a regulator of intestinal immune homeostasis. In this regard, the interaction of epithelial cells with the microbiota has the key role of mediator of the cross-talk between the epithelium and other cell types in the mucosa (Darab et al. 2011). Indeed, intestinal epithelium cells (IECs) express a wide variety of pattern recognition receptors (PRR) to detect the presence of microbes. The well-characterized PRR expressed by epithelial cells include principally members of the Toll-like (TLR) family, which can trigger expression of a surprising diversity of chemokines, cytokines and effectors of innate and inflammatory response (Sieling and Modlin 2002; Abreu et al. 2003). Immunohistochemical techniques have shown that TLR2 and TLR4 are expressed at low levels by IEC in the human colon tissues of healthy individuals; TLR2 plays a major role in the detection of Gram-positive bacteria via recognition of cell wall PAMPs, including lipoteichoic acid, lipoproteins, and peptidoglycan (Abreu et al. 2005; Kelly and Conway 2005), while TLR4 plays a role in the intestinal mucosal host defense against Gram-negative bacteria and is essential for LPS detection (Vinderola et al. 2005). The intestinal microbiota is largely mutualistic in nature and important for human health. Apart from its well-established role in nutrition, it is important in development of the immune system and maintenance of homeostasis of tolerance and immunity (Himanshu et al. 2001). Administration of indigenous, non-pathogenic probiotic bacteria is a promising strategy to improve immune homeostasis and to maintain host health by normalizing existing undesired immune responses, as in the case of allergy or autoimmune disease (Vinderola et al. 2005; Adams 2010). Epithelial cells from the small and large intestines make a major distinction between probiotic and pathogens. This distinction comprises the type of cytokines released and the magnitude of the response (Peyrin-Biroulet et al. 2006).

The interest in the innate immune response in inflammatory bowel diseases has led to evaluate the role of defensins, part of a family of antimicrobial peptides with direct bactericidal activity against a wide variety of bacteria (Peyrin-Biroulet et al. 2006; Marian et al. 2009). Human β-defensins (HBDs) are small cationic antimicrobial peptides synthesized by the epithelium to counteract bacterial adherence and invasion. HBDs are expressed in various tissues throughout the body; the best characterized gut defensin is the HBD-2, an inducible peptide, synthesized and secreted by the epithelium, with a strong antimicrobial activity (Vora et al. 2004). Unlike HBD-1, which is produced constitutively, HBD-2 is expressed after adequate stimulation by cytokines and/or bacterial components in epithelial tissues and mononuclear phagocytes. Some probiotic lactobacilli have been shown to induce HBD-2, but they are not affected by the antimicrobial effect of HBD-2, which appear to be specific for pathogens (Schlee et al. 2007). Moreover, a further effect of probiotics on the mucosal barrier function is the ability to induce production of some anti-inflammatory cytokines (e.g. IL-10, TGF-beta) and transient production of pro-inflammatory cytokines (e.g. IL-6) (Boirivant and Strober 2007; Li et al. 2006).

The effect of probiotics on modulation of immune response is strain dependent, mainly based on different molecules expressed on cell surface, needed for the cross-talk with epithelial

cells. Among these molecules, adhesins have a critical role in colonization of the intestine, in protection against pathogens trough competitive exclusion, and in development of biofilms, important for persistence of probiotic strains in the colon (Macfarlane 2008). Lactic acid bacteria (LAB) of the genus *Lactobacillus* and *Bifidobacterium*, traditionally present in dairy products, have been used for treatment and prevention of gut diseases, and the beneficial effects of some probiotic LAB strains have been assessed by clinical trials (Gareau et al. 2010; Ahrne and Hagslatt 2011). Among these, some strains of *Lactobacillus plantarum*, one of the most predominant species in the human microbiota of healthy individuals, were demonstrated to be good performing probiotic microorganisms (van Baarlen et al. 2009; Kaushik et al. 2009; Lonnermark et al. 2010). We have previously shown adhesion of *L. plantarum* LM3 to Caco-2 cells, and identified enolase as a surface-expressed protein involved in adhesion to fibronectin and to plasminogen (Castaldo et al. 2009; Vastano et al. 2013). Enolases are highly conserved proteins, with an essential role in central metabolism and with moonlighting functions in many microorganisms and in different eucariotic cell types. Surface-expressed enolases are involved in pathogenesis of many microorganisms, either in the role of adhesion molecules for different host receptors, or as molecules involved in the host immuneresponse (Veiga-Malta et al. 2004; Adrian et al. 2015).

The aim of this study was to evaluate the potential immunomodulatory effects of *L. plantarum* LM3 and its adhesion-defective mutant LM3-CC1, carrying a deletion in the *enoA1* gene, coding the enolase EnoA1. The analysis was performed by determining the expression level of some immunomodulatory molecules on Caco-2 cells exposed to *L. plantarum* wild type and mutant strain. Moreover, the role of the surface expressed enolase in the *L. plantarum* ability to develop biofilm, the sessile form adopted by microorganisms to persist in the colon, was also evaluated.

2 Methods

2.1 Cell Lines, Media and Bacterial Strains

The human colon adenocarcinoma Caco-2 cells, (from the American Type Culture Collection ATCC), were maintained in Dulbecco's Modified Eagle's Medium (DMEM). Growth media were supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS), 1 % (v/v) non-essential amino acids, and a standard mixture of antibiotics (100 U ml − 1 penicillin, 100 μg ml^{-1} streptomycin) and 2 mmol l-glutamine at 37 °C under an atmosphere of 5 % CO_2. All reagents were purchased from Gibco-BRL (UK). Caco-2 cells differentiated into enterocytes after 15–20 days in culture. The cells were grown as monolayers in 75 cm^2 flasks (Greiner, Frickenhausen, Germany) at 37 °C in a 5 % CO_2-95 % air atmosphere with 90 % humidity. *L. plantarum* LM3 (Muscariello et al. 2013) and LM3-CC1 (Veiga-Malta et al. 2004) strains were cultured at 30 °C in MRS broth supplemented with either 0.2 % or 2 % glucose. When needed, erythromycin (5 μg ml^{-1}) was added to *L. plantarum* LM3-CC1 cultures.

2.2 Stimulation of Caco-2 Cells

Caco-2 cells were cultured in six-well plates (Becton Dickinson GmbH, Heidelberg, Germany) at a concentration of 10^6 cells/ml for 12 h. Caco-2 cells were incubated with *L. plantarum* LM3 or LM3-CC1 (100 bacteria/cell) for 3, 6, and 24 h in 5 % CO_2 at 37 °C. Infected monolayers were centrifuged at 6000×*g* for 5 min to favor adhesion. After the specific time of treatment, infected monolayers were washed three times with DMEM to remove non-adherent bacteria. Untreated Caco-2 cells served as controls. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (ATCC, Manassas, USA),

according to the manufacturer's protocol. The culture media were then collected, cells were washed twice with phosphate-buffered saline (PBS) and harvested with High Pure RNA Isolation Kit (Roche Diagnostics, Milano, Italy) from Caco-2 cells infected or not with *L. plantarum*.

2.3 Real-Time PCR

To study expression of TLR2, TLR4, HBDs, IL-6, TGF-β and IL-10 in Caco-2 cells, Real-Time PCR was performed on RNA extracted from the cells challenged with *L plantarum* LM3 and LM3-2. Real-time PCR analyses were performed in a fluorescence temperature cycler (LC Fast Start DNA Master SYBR Green, Roche Diagnostics) (LightCycler 2.0 Instrument, GmbH, Mannheim, Germany) according to the manufacturer's instructions. cDNA corresponding to 10 ng of RNA served as a template in a 20 μl reaction mixture containing 3 mM $MgCl_2$, 0.5 μM of each primer and 1X LightCycler-FastStart DNA Master SYBR Green I mix. Sequences of the oligonucleotides used for amplification and related programs are shown in Table 1. At the end of each run, melting curve profiles were achieved by cooling the sample to 65 °C for 15 s and then heating slowly at 0.20 °C/s up to 95 °C, with continuous measurement of fluorescence to confirm amplification of specific transcripts. Cycle-to-cycle fluorescence emission readings were monitored and analyzed using LightCycler software (Roche Diagnostics GmbH). Melting curves were generated after each run to confirm amplification of specific transcripts. As an internal control gene we used the β-actin coding gene, one of the most commonly used housekeeping genes. All reactions were carried out in triplicate, and the relative expression of a specific mRNA was determined by calculating the fold change relative to the β-actin control. The fold change of the test gene mRNA was obtained with a Biorad software, using the efficiency of each primer as calculated by the dilution curve. $\Delta Ct =$ the difference of Ct between stimulated and control samples (Valaesk and Repa 2005).

The specificity of the amplification products was verified by subjecting the amplification products to electrophoresis on a 2 % agarose gel and visualization by ethidium bromide staining.

Table 1 Primers used in the study

Gene	Sequence sense and antisense	Conditions	bp
HBD-1	5′-TTG TCT GAG ATG GCC TCA GGT GGT AAC-3′	15″ at 95 °C, 5″ at 60 °C,	200
	5 -TTT CAC TTC TGC GTC ATT TCT TCT GG- 3′	10″ at 72 °C for 45 cycles	
HBD-2	5′- GGATCCATGGGTATAGGCGATCCTGTTA -3′	5″ at 94 °C, 6″ at 63 °C,	198
	5′- AAGCTTCTCTGATGAGGGAGCCCTTTCT −3′	10″ at 72 °C for 50 cycles	
HBD-3	5′ -AGC CTA GCA GCT ATG AGG ATC-3′	15″ at 95 °C, 5″ at 60 °C,	206
	5′ -CTT CGG CAG CAT TTT GCG CCA-3′	10″ at 72 °C for 45 cycles	
TLR-2	5′-TCGGAGTTCTCCCAGTTCTCT-3′	30″ at 95 °C, 30″ at 59 °C,	175
	5′-TCCAGTGCTTCAACCCACAA-3′	30″ at 72 °C for 50 cycles	
TLR-4	5′-CGTGGAGACTTGGCCCTAAA-3′	30″ at 95 °C, 30″ at 59 °C,	301
	5′-TTCACACCTGGATAAATCCAGC-3′	30″ at 72 °C for 50 cycles	
IL-6	5′-ATGAACTCCTTCTCCACAAGCGC-3′	5″ at 95 °C, 13″ at 56 °C,	628
	5′-GAAGAGCCCTCAGGCTGGACTG-3′	25″ at 72 °C for 40 cycles	
IL-10	5′-CTTTAAGGGTTACCTGGGTTGCCAAG-3′	5″ at 95 °C, 9″ at 60 °C, 18″	223
	5′-ATTAAAGGCATTCTTCACCTGCTCCAC-3′	at 72 °C for 35 cycles	
TGF-β_1	5′-CCGACTACTACGCCAAGGAGGTCAC-3′	5″ at 95 °C , 8″ at 60 °C, 16″	439
	5′-AGGCCGGTTCATGCCATGAATGGTG-3′	at 72 °C for 35 cycles	
β-actin	5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′	33 cycles 94 °C for 30″	661
	5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′	63 °C for 30″, 72 °C for 30″	

2.4 Microtiter Plate Assay of *L. plantarum* Biofilm Formation (for Biofilm Quantization)

The microtiter plate assay measures the level of cells adhering to the surface of microtiter plate wells. The assay was performed as described by Hamon and Lazazzera (2001), with the following modifications. Cells were grown at 30 °C in MRS medium supplemented with 2 % or 0.2 % (wt/vol) glucose under static conditions. Overnight cultures of *L. plantarum* LM3 (wt) and LM3-CC1 were diluted in fresh medium to obtain optical density (OD_{600}) of 0.05. Two hundred ml of each diluted cell suspension were inoculated into the wells of a 96-well (flat-bottom) cell culture plate (Falcon 35-3072). Wells containing un-inoculated growth medium were used as negative controls. Plates were incubated at 30 °C for 24 h in static condition. Media and unattached cells were removed by rinsing with 200 ml of sterile wash buffer (150 mM $(NH_4)_2SO_4$, 100 mM potassium-phosphate buffer pH 7.0, 34 mM $Na_3C_6H_5O_7$, 1 mM $MgSO_4$). Adherent bacteria were stained with 200 µl of 1 % (wt/vol) crystal violet for 15 min at room temperature. After two rinses with 200 µl of sterile distilled water each time, the bound dye was extracted from the stained cells by using 200 µl of 80 % ethanol, 20 % acetone. Biofilm formation was quantified by measuring the OD_{570} for each well using a Bio-Rad model 680 microplate reader.

2.5 Statistical Analysis

The significance of the differences in the results of each test compared to the relative control values was determined with the Student t-test. Values of $P < 0.05$ were considered statistically significant. The data are presented as means ± standard deviation (SD) of three independent experiments.

3 Results

3.1 Expression of TLR-2 and TLR-4 in Caco-2 Cells Upon Exposure to *L. plantarum* LM3 and LM3-CC1

To study the immunomodulatory effect of *L. plantarum* on Caco-2 cells we first determined the expression of the TLR-2 and TLR-4 mediators. The LM3 wild type induced of about 3 fold the expression of TLR-2 in Caco-2 cells after 3 h of exposure; the effect decreased to 2.3 fold after 6 h, to reach basal levels upon 24 h of exposure (Fig. 1, panel A). Treatment of Caco-2 cells with the LM3-CC1 strain failed to induce TLR-2 expression as monitored up to 24 h exposure (Fig. 1, panel A).

Expression of TLR-4 was induced of 1.6 fold after 3 h exposure to LM3 wild type, and decreased to basal levels upon 24 h exposure (Fig. 1, panel B); *L. plantarum* LM3-CC1 stimulated a similar level of TLR-4 expression in Caco-2 cells (Fig. 1, panel B).

3.2 *L. plantarum* LM3-Induced HBD-2 Expression in Caco-2 Cells

To determine whether human colon epithelial cell lines constitutively express β-defensins, RNAs from un-stimulated Caco-2 cells were analysed by Real Time PCR using HBD-1, HBD-2 and HBD-3 specific primers. Caco-2 cells constitutively expressed a low level of mRNA for HBD-1 and HBD-3, whereas there was little if any constitutive expression of HBD-2 mRNA (data not shown).

When Caco-2 cells were exposed to *L. plantarum* LM3, HBD-2 expression was induced more than 3 times after 6 h of exposure, to reach again the basal level of expression upon 24 h treatment (Fig. 2). Exposure of Caco-2 cells to *L. plantarum* LM3-CC1 mutant strain produced a minor effect on HBD-2 expression,

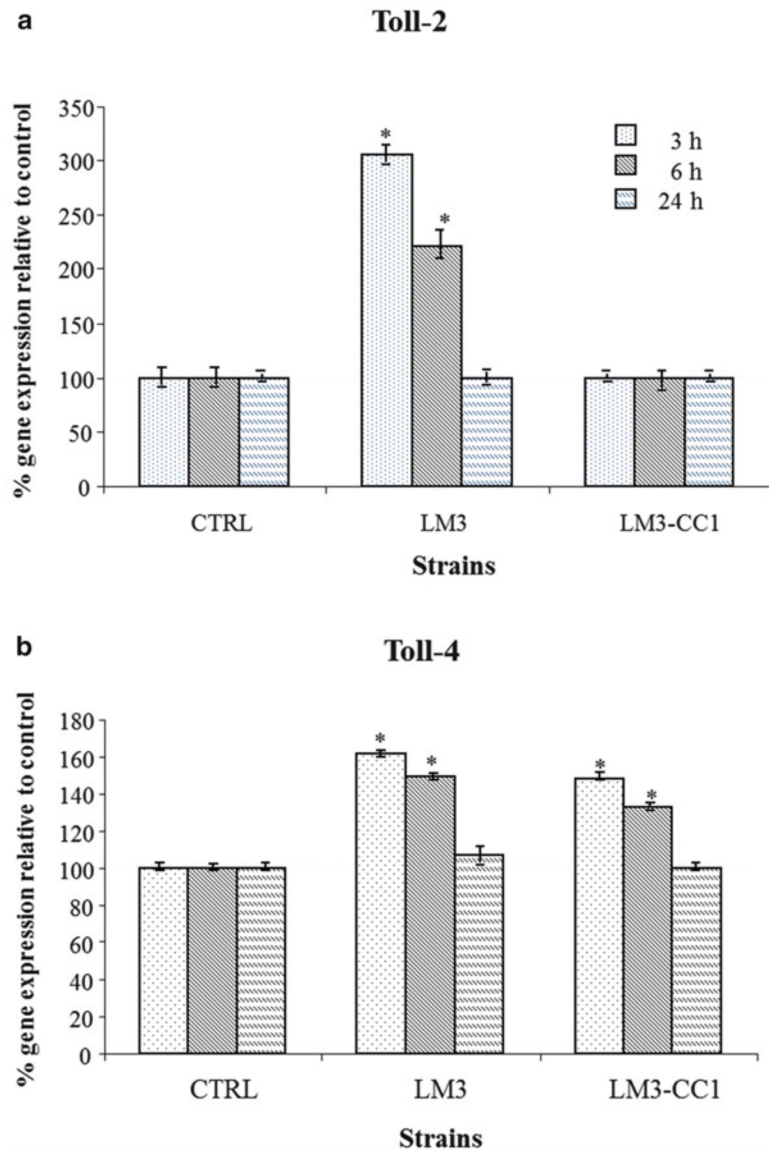

Fig. 1 Expression levels of *TLRs* genes in Caco-2 cells in the presence of *L. plantarum* LM3 and LM3-CC1. Comparison of the expression of TLR2 (**a**) and TLR4 (**b**) in Caco-2 cells after 3 h, 6 h, and 24 h exposure to *L. plantarum* LM3 or LM3-CC1. Expression levels are reported exposure. Data are expressed as percentage of the relative mRNAs in each sample compared to un-stimulated Caco-2 cells (CTRL), arbitrarily assigned as 100 %. The data are the mean values of three independent experiments (*$p < 0.05$ relative to control sample)

namely induction of only 2 times after 6 h treatment, to reach basal levels within 24 h exposure (Fig. 2).

3.3 *L. plantarum* LM3-Dependent Expression of Cytokines in Caco-2 Cells

We extended the analysis of the immunomodulatory effect determined by *L. plantarum* LM3 and LM3-CC1 to the expression of cytokines in Caco-2 cells. Expression of the pro-inflammatory IL-6 was transiently induced more than five times after 6 h exposure to LM3 cells, to decrease to almost basal levels after 24 h treatment (Fig. 3, panel A). Exposure of Caco-2 cells to LM3-CC1 determined a much lower level of induction, being the expression of IL-6, after 6 h exposure, only twice as much the level detected in un-stimulated Caco-2 cells (Fig. 3, panel A). Moreover, expression of anti-

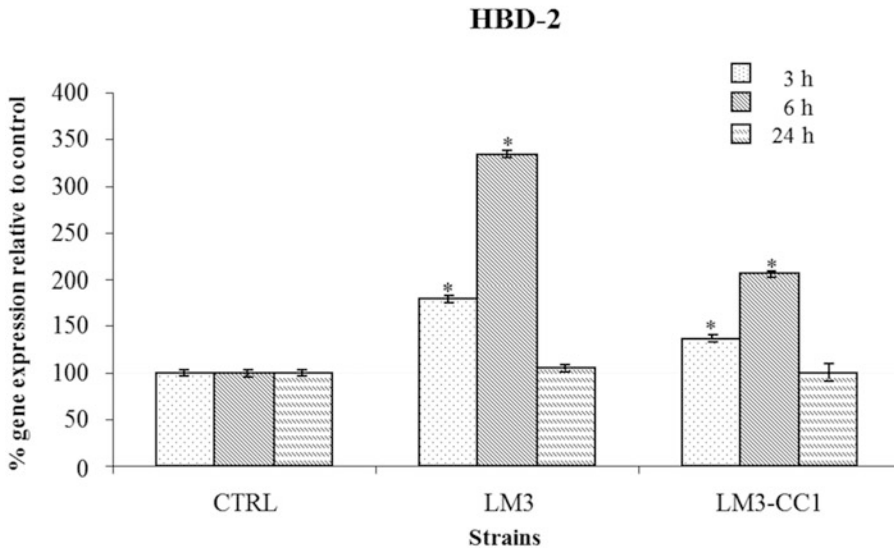

Fig. 2 Expression levels of *HBD-2* genes in Caco-2 cells in the presence of *L. plantarum* LM3 and LM3-CC1. Comparison of the expression of HBD-2 in Caco-2 cells after 3 h, 6 h, and 24 h exposure to *L. plantarum* LM3 or LM3-CC1. Expression levels are reported exposure. Data are expressed as percentage of the relative mRNAs in each sample compared to un-stimulated Caco-2 cells (CTRL), arbitrarily assigned as 100 %. The data are the mean values of three independent experiments (*p $<$ 0.05 relative to control sample)

inflammatory cytokines, namely IL-10 and TGFβ, were induced in Caco-2 cells exposed to *L. plantarum* LM3, reaching an expression of about 3,5 and 4 fold, respectively, after 24 h of treatment (Fig. 3, panels B and C). Exposure to LM3-CC1 produced an increase of IL-10 and TGFβ of 1.8 and 2.4 fold, respectively, after 3 h of treatment, and the level of induction remained mostly stable up to 24 h of treatment (Fig. 3, panels B and C).

3.4 Surface Expressed EnoA1 Affects Biofilm Development in *L. plantarum*

The ability of a microorganism to develop biofilm in the gut environment plays a role for its persistence in the colon, being therefore considered a prerequisite for good performing probiotic strains (Ahrne and Hagslatt 2011). Development of *L. plantarum* LM3 biofilm was tested at different pH values, ranging from 4.0 to 8.0. Respect to the positive control, namely biofilm developed at pH 7.0, no significant variation was observed at alkaline pH, which is a condition encountered in the colon, while about 40 % reduction was observed at pH 4.0 (data not shown). A comparative analysis between the two strains under study was then performed for the ability to develop biofilm at pH 7.0. A decrease of more than three fold was found in the amount of biofilm developed by *L. plantarum* LM3-CC1 respect to the wild type (Fig. 4), suggesting the involvement of the EnoA1 adhesin in this process.

4 Discussion

The gut microbiota is undoubtedly important in supporting a functional yet balanced immune system; processes leading to this balance can be emulated by transiently colonizing the gastrointestinal tract with appropriate strains of microbes that are delivered orally as probiotics and that influence the host microbiota and immune functions of the immune cells associated with the gut (Abreu et al. 2003).

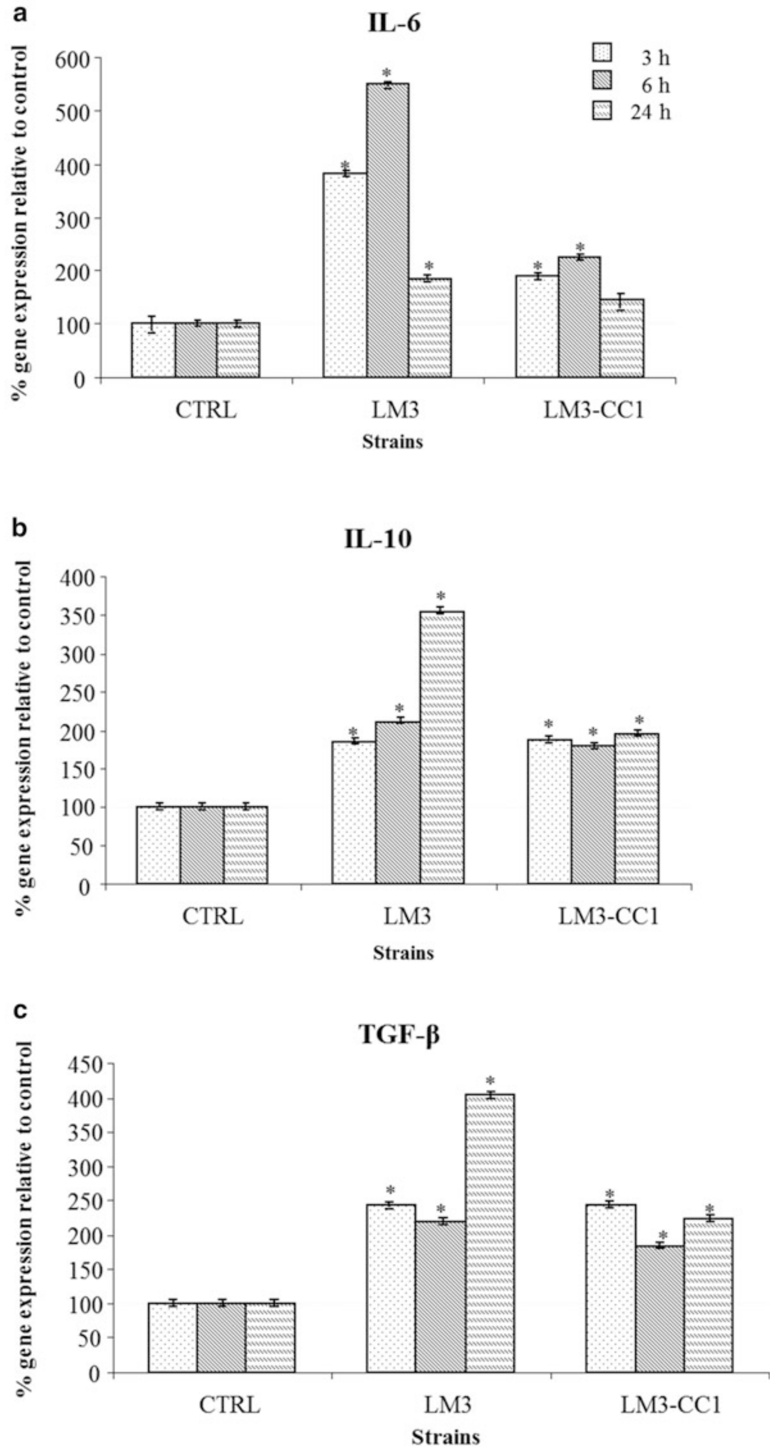

Fig. 3 Expression levels of pro-inflammatory and anti-inflammatory cytokines genes in Caco-2 cells in the presence of *L. plantarum* LM3 and LM3-CC1. Comparison of the expression of IL-6 (**a**), IL-10 (**b**), and TGF-β (**c**) after 3 h, 6 h, and 24 h exposure to *L. plantarum* LM3 or LM3-CC1 Expression levels are reported exposure. Data are expressed as percentage of the relative mRNAs in each sample compared to un-stimulated Caco-2 cells (CTRL), arbitrarily assigned as 100 %. The data are the mean values of three independent experiments (*$p < 0.05$ relative to control sample)

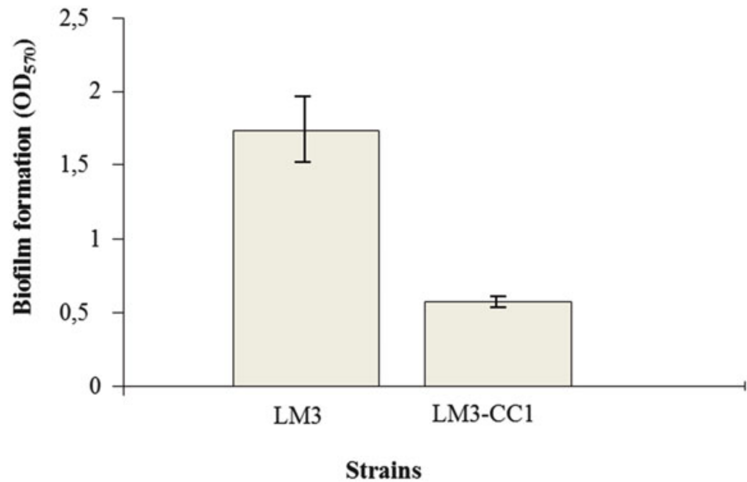

Fig. 4 Quantitative analysis of biofilm formation performed on *L. plantarum* LM3 and LM3-CC1. Biofilm assay was performed on cultures of each strain grown to stationary phase (24 h) in MRS medium containing 0,2 % glucose. Results are the average of three independent experiments. The error bars represent standard deviation of the mean

Indeed, IEC is continually confronted by a variety of commensal bacteria and pathogen-associated molecular patterns (PAMPs). However, in the absence of pathogens, the lamina propria maintains a controlled state of inflammation through the development of a careful system of controlled TLR expression (Melmed et al. 2003). The expression of TLRs has been reported to increase during the course of infections or inflammatory bowel diseases (Cario and Podolsky 2000; Faure et al. 2001). Although TLR2 is normally present in epithelial cells, it plays a limited role in inflammation. It may be activated under conditions in which bacterial cell wall concentrations within the intestine are pathologically high (Naik et al. 2001; Otte et al. 2004). TLR4 is instead expressed at lower basal level respect to TLR2 in epithelial cells, and plays a role in the intestinal mucosal host defense against Gram-negative bacteria, being essential for LPS detection (Vinderola et al. 2005). Probiotic strains have been shown to counteract the inflammation process stimulated by pathogens with induction of anti-inflammatory cytokines secretion by a TLR2 dependent pathway, and induction of pro-inflammatory and anti-inflammatory cytokines secretion by a TLR4 dependent pathway; however, probiotics were shown to stimulate higher levels of TLR2 respect to TLR4 in epithelial cells (Bermudez-Brito et al. 2012; Villena and Kitazava 2014). Moreover, some probiotic lactobacilli induce HBD-2 expression in epithelial cells, but they are not affected by the antimicrobial effect of HBD-2, which appears to be specific for pathogens (Schlee et al. 2007).

In this work we studied the immunomodulatory properties of *L. plantarum* LM3, by analysing expression of TLR2 and TLR4 mediators, IL-6 pro-inflammatory and IL-10 and TGF-beta anti-inflammatory cytokines, and the HBD-2 defensin in Caco-2 cells stimulated with *L. plantarum*.

Our results suggest that *L. plantarum* LM3 may use TLR-2, and to a lower extent TLR-4, to send immune signals to IEC, and the immune signals released would be closer to those of inflammation. This represents a mechanism that may impact on host immunity and disease development (Vinderola et al. 2005). Moreover, we showed that *L. plantarum* LM3 is able to induce HBD-2 expression. The time-dependence experiments showed a similar pattern as already described in previous studies with a maximum of HBD-2 induction after 6 h of incubation (Wehkamp et al. 2004). The interest in antibacterial peptides, as an innate mechanism of defense against bacteria, has increased in recent years. Antimicrobial peptides (AMPs) act mainly by damaging or destabilizing cytoplasmic membrane or at cytoplasmic level by inhibiting essential cellular processes. Indeed, recent evidences

suggest that probiotic bacteria may stabilize gut barrier function against pathogenic luminal bacteria through the induction of antimicrobial peptides, such as human β-defensins (HBDs) (Schlee et al. 2007; Schlee et al. 2008; Hugo et al. 2010). In particular, HBD-2 has been shown to be induced in the gut, and up-regulated in inflammatory bowel disease (Wehkamp et al. 2004; Schlee et al. 2008).

We also showed that *L. plantarum* LM3 induces transient expression of IL-6 in Caco-2 cells. The transient production of pro-inflammatory cytokines has been already reported for probiotic strains (Ukena et al. 2005; Ruiz et al. 2005). Indeed, probiotic bacteria induce the production of IL-6, necessary for T cell-independent clonal expansion of IgA producing-B cells, thus protecting epithelia from attachment of pathogens (Boirivant and Strober 2007). Moreover, our results indicate that IL-10 and TGF-beta anti-inflammatory cytokines were induced, reaching about four-fold increase after 24 h exposure. IL-10 has pleiotropic effects in immunomodulation and inflammation, being an essential immune-regulator in the intestinal tract; it is able to inhibit the synthesis of pro-inflammatory cytokines as well as to suppress the antigen presentation capacity of antigen presenting cells (Grimbaldeston et al. 2007). TGF-beta is involved in the control of tolerance through modulation of immune-cells proliferation, thus regulating initiation and resolution of inflammatory response (Li et al. 2006). Indeed, transient expression of pro-inflammatory and expression of anti-inflammatory cytokines in epithelial cells challenged with probiotics have been related to beneficial effects of these bacterial strains, representing a mechanism for control of pathogen invasions and a way for resolution of inflammatory response (Li et al. 2006; Villena and Kitazava 2014).

The ability of a microorganism to interact with host epithelial cells has been related to expression of proteins on cell surface. Among these, a large number of enzymes of the central metabolism in bacteria were shown to have additional functions in dependence of their localization; in particular some glycolytic enzymes were shown to be expressed on cell surface and to be involved in binding to host cells and proteins (Henderson and Martin 2013; Kainulanen and Korhonen 2014). In previous works we have shown that the EnoA1 alfa-enolase of *L. plantarum* LM3 is surface expressed and mediates interaction with fibronectin, plasminogen and Caco-2 cells (Veiga-Malta et al. 2004; Adrian et al. 2015). By means of comparative analysis between *L. plantarum* LM3 and its isogenic mutant strain *L. plantarum* LM3-CC1, carrying a deletion in the *enoA1* gene, here we show that EnoA1 is involved in stimulation of TLR2 expression; indeed, Caco-2 cells challenged with LM3 wild type showed a three fold increase in TLR2 expression, while cells exposed to the LM3-CC1 mutant strain showed the same basal level found in the control sample. On the contrary, the absence of EnoA1 did not affect stimulation of TLR4 in Caco-2 cells challenged with LM3-CC1. Stimulation of cytokines and HBD2 is only partially affected by the absence of EnoA1, probably due to their already described double dependence on TLR2 and TLR4 pathways (Vora et al. 2004; Bermudez-Brito et al. 2012). We can not exclude that additional TLR pathways may be involved in *L. plantarum* immunomodulation.

We also found that the absence of EnoA1 lowered the ability to develop biofilm in *L. plantarum* LM3-CC1. Adhesins have a critical role in development of biofilms, important for persistence of probiotic strains in the colon (Ahrne and Hagslatt 2011). Moreover, it was shown that *Lactobacillus reuteri* biofilms have a higher protective effect on human THP-1 cells respect to the planktonic counterpart (Jones and Bersalivic 2009). Effects of treatments based on probiotics administration to counteract biofilm-associated infections have been recently reviewed (Vuotto et al. 2014).

In summary, here we show evidence for the role of surface expressed enolase in immunostimulation of Caco-2 cells exposed to *L. plantarum* LM3. Enolase has been described as an important antigen involved in virulence of many pathogens (Henderson and Martin 2013; Pancholi 2001; Dinis et al. 2009). Knowledge about enolase-

mediated activation of pathways involved in protective immunomodulation triggered by probiotics may give an insight on their competitive exclusion mechanisms against pathogens.

Competing Interests The authors declare that they have no competing interests.

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Partial Protection Induced by 2011–2012 Influenza Vaccine Against Serologically Evidenced A(H3N2) Influenza Virus Infections in Elderly Institutionalized People

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Abstract

Ninety-two institutionalized elderly subjects were vaccinated with trivalent influenza inactivated vaccine available for the 2011–2012 season, characterized by a prevalent circulation of A(H3N2) influenza viruses (A/Victoria/208-clade) presenting antigenic and genetic patterns different from the A(H3N2) vaccine component (A/Perth/16/2009-clade). Haemagglutination inhibiting (HI) antibody titers were determined in sera collected before, 1 and 6 months after vaccination and patients were considered positive for serological evidence of recent infection if they had a seroconversion on comparing HI titers found in sera collected 1 and 6 months after vaccination. No seroconversions were found against A(H1N1) and B vaccine components. Instead 17 volunteers seroconverted against all or at least some of the different A(H3N2) antigens examined, i.e. the 2011–2012 (A/Perth/16/2009) and the 2012–2013 (A/Victoria/361/2011) vaccine strains and four drifted viruses belonging to the A/Victoria/208-clade circulating in the area were the elderly people were living. The results obtained suggest that influenza infections in the vaccinated volunteers might be due both to a poor match between vaccine and circulating A(H3N2) viruses, since 1 month after vaccination 15 of the 17 volunteers had post-vaccination HI titers considered protective ($\geq$40) against the A(H3N2) vaccine antigen, but not always against the epidemic strains, and to a waning of vaccine induced immune response, since 6 months after vaccination HI titers of non-infected volunteers were found to be decreased as compared with those found 1 month after vaccination.

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Influenza virus • Vaccination • Protection • Antigenic drift

1 Short Data Report

Influenza vaccine effectiveness varies according to the match between the yearly selected vaccine strains and circulating viruses (Kissling et al. 2013). The 2011–2012 winter season, as the present 2014–2015 winter season (Broberg et al. 2015), was characterized by a dominant circulation of A(H3N2) influenza viruses clustered into several genetic groups (A/Victoria/208-clade) that were reported to be antigenically and genetically distinct from the clade of the vaccine strain (A/Perth/16/2009-clade), resulting in a change in the WHO recommended A(H3N2) vaccine strain for 2012–2013 in the northern hemisphere (World Health Organization 2012).

Several test-negative case-control studies estimated vaccine effectiveness (VE) against medically attended influenza like-illness (ILI) in the 2011–2012 winter season. A low to moderate VE against ILI with laboratory confirmed influenza A(H3N2) virus infection was found in the late season especially in the older individuals and it was postulated that this could be due to a combination of poor match between vaccine and circulating viruses and of a waning of protection (Pebody et al. 2013).

In this study we obtained results supporting this hypothesis analyzing both the efficacy of vaccination, as measured by serological evidence of recent influenza infections, and the persistence of the antibody response 6 months after vaccination in a group of elderly volunteers living in nursing homes in Italy. Previous observations evidenced that older people living in seniors' housing have a higher risk of influenza infection compared with independent-dwelling older people even though vaccination rates are similar (McElhaney 2002). Moreover, studies of the effects of influenza vaccination based on seroconversion are less limited than those based on clinical diagnosis confirmed by laboratory identification of influenza virus, since they can evidence also influenza infections without clinical symptoms.

The study was conducted according to Good Clinical Practices and the declaration of Helsinki. After obtaining informed consent, 92 volunteers aged ≥65 years living in nursing homes of Umbria (a small region of central Italy) were immunized in November 2011 with trivalent influenza seasonal vaccines. Two implemented vaccines licensed for individuals aged ≥65 years were used: 52 volunteers were vaccinated with Fluad® (Novartis Vaccines, Italy), a subunit vaccine adjuvanted with MF59 and administered intramuscularly, and 40 with Intanza® 15 mcg (Sanofi-Pasteur MSD, France) a split vaccine administered intradermally.

Serum samples, obtained from each volunteer before, 1 and 6 months after vaccination, were analyzed by hemagglutination inhibiting (HI) assay using turkey erythrocytes (0.5 %) and sera pre-treated with receptor-destroying enzyme and heat-inactivated at 56 °C for 30 min to remove non-specific inhibitors (Harmon 1992). The antigens used were the three strains included in the 2011–2012 influenza vaccine (A/Perth/16/2009 (H3N2), A/California/7/2009 (H1N1) and B/Brisbane/60/2008), the new A(H3N2) strain included in the composition of 2012–2013 influenza vaccine (A/Victoria/361/2011), and four A(H3N2) field viruses (A/Perugia/06/2012, A/Perugia/20/2012, A/Perugia/44/2012 and A/Perugia/50/2012) isolated in the winter 2011–2012 from people with ILI living in the region where the nursing homes were located. The genetic characteristics of the field viruses, obtained by sequencing the complete domain 1 of the haemagglutinin (HA) gene with specific primers, were previously published (Camilloni et al. 2014) and all the four viruses belonged to the new A/Victoria/208/2009 clade.

A serological evidence of recent influenza infection after vaccine administration was made

on comparing HI titers found in sera collected 6 and 1 months after vaccination. Vaccinated volunteers were considered positive if they had a seroconversion (a fourfold or greater increase in titer in seropositive subjects or from <10 to ≥ 40 in seronegative volunteers). Although in seropositive subjects before vaccination the seroconversion parameter may underestimate the rate of infections, no seroconversions were observed against A (H1N1) and B vaccine antigens (data not reported). On the contrary positive results were found against the A(H3N2) antigens examined. Totally, 17 of the 92 volunteers enrolled (18.5 %) seroconverted, 11 vaccinated with the MF59 adjuvanted vaccine and 6 with the intradermally administered vaccine. Table 1 shows the increases in HI titers observed in each volunteer. In most instances the infections were evidenced using the 2011–2012 and 2012–2013 A(H3N2) vaccine strains and the four epidemic A(H3N2) viruses as antigens.

To try to understand the reasons of the observed infections in vaccinated people we examined different parameters.

The possibility of a non-adequate response induced by the vaccination was analyzed by considering in the 17 vaccinated and infected people the achievement against the different A(H3N2) virus strains studied of at least 1 of the 3 immunological criteria of the European Medicine Agency (EMA) requirements (seroprotection rate (percentage of subjects achieving an HI titer ≥ 40) ≥ 60 %; Geometric Mean Titer Ratio (GMTR) (ratio of post- to pre-vaccination GMT) ≥ 2; or seroconversion rate ≥ 30 %) 1 month after vaccination (Katz et al. 2011). As shown in Table 2, similar responses and fulfillment of all the 3 EMA requirements were found both against the A(H3N2) 2011–2012 and the new A(H3N2) 2012–2013 vaccine antigens. Two of the three criteria (seroprotection rate and GMTR) were reached using as antigens 2 of the 4 drifted strains (A/PG/06/2012 and A/PG/50/2012), whereas against the other two (A/PG/20/2012 and A/PG/44/2012) none of the criteria was fulfilled. The values found against the new 2012–2013 A(H3N2) vaccine strain and against the first two drifted strains tended to be lower as compared with those against the 2011–2012 vaccine strain, whereas the values found against the latter two drifted strains were in most instances significantly lower than those against the 2011–2012 and the 2012–2013 A(H3N2) vaccine strains.

The second point we examined was the achievement of protective antibody titers, an important goal of vaccination. Although an HI titer of at least 40 is considered as 50 % protective, it is not clear if the seroprotective HI titer of 40 applies to protection in older adults and in other groups at high risk for complications of influenza infection (Katz et al. 2011). Moreover, since the correlation between increasing serum anti-HA antibody titer and reducing occurrence of influenza has been repeatedly demonstrated (De Jong et al. 2003; Couch 2003; Coudeville et al. 2010), we examined the percentages of vaccinated and infected people reaching 1 month after vaccination an HI titer ≥ 40 or ≥ 160, considered more protective (Table 2).

HI titers ≥ 40 were found in an high percentage of volunteers against the 2011–2012 and against 2012–2013 A(H3N2) vaccine strains, (respectively in 88.2 and 70.6 %) and against two of the epidemic strains studied (64.7 % against A/PG/06/2012 and 70.6 % against A/PG/50/2012), whereas the percentages against the other two epidemic strains were low and did not reach 60 % (47.0 % against A/PG/44/2012 and 17.6 % against A/PG/20/2012).

The percentages of the 17 infected people showing 1 month after vaccination an HI titer ≥ 160 were reduced, as compared with those for HI titer ≥ 40, to 35.3 and 23.5 % against the 2011–2012 and 2012–2013 A(H3N2) vaccine antigens respectively. Very few volunteers showed HI titers ≥ 160 against 2 of the 4 epidemic A(H3N2) strains (1 against A/PG/06/2012 and 2 against A/PG/50/2012) and nobody against the other two epidemic strains (A/PG/20/2012 and A/PG/44/2012).

Further considerations derived from the HI test used in the study. Although HI titers were previously found to correlate in many instances well with those detected with neutralization (Nt) assay, some dissociation may exist. HI detects only antibodies directed against viral HA globular head that act by preventing erythrocyte agglutination, whereas Nt detects functional antibodies that

Table 1 Seroconversions against different A(H3N2) influenza virus antigens [A/Perth/16/09 (2011–2012 vaccine antigen), A/Victoria/361/2011 (21012–2013 vaccine antigen) and four epidemic drifted strains (A/PG/06/2012, A/PG/20/2012, A/PG/44/2012, A/PG/50/2012)] comparing HI titers in sera collected before, 1 and 6 months after 2011–2012 vaccination from 17 elderly institutionalized volunteers

	A/Perth/16/2009			A/Victoria/361/2011			A/PG/06/12			A/PG/20/12			A/PG/44/12			A/PG/50/12		
Case (Vaccine[a])	Pre	1 mo	6 mo	Pre	1 mo	6 mo	Pre	1 mo	6 mo	Pre	1 mo	6 mo	Pre	1 mo	6 mo	Pre	1 mo	6 mo
1 (F)	5	5	160[c]	10	10	40[c]	10	10	40[c]	10	10	40[c]	10	20	80[c]	20	20	80[c]
2 (F)	5	20	320[c]	5	10	160[c]	5	10	40[c]	5	5	40[c]	5	20	80[c]	10	20	80[c]
3 (F)	5	40[b]	1280[c]	10	40[b]	320[c]	20	40	160[c]	5	5	160[c]	20	20	160[c]	40	40	160[c]
4 (I)	5	80[b]	320[c]	5	80[b]	320[c]	10	40[b]	320[c]	5	20	320[c]	5	40[b]	320[c]	5	20	320[c]
5 (F)	5	640[b]	1280[c]	5	160[b]	1280[c]	20	40	320[c]	5	80[b]	1280[c]	5	80[b]	640[c]	5	160[b]	1280[c]
6 (F)	10	40[b]	320[c]	20	40	80	5	20	80[c]	5	10	40[c]	5	20	80[c]	10	20	80[c]
7 (I)	10	40[b]	160[c]	5	40[b]	80	40	40	160[c]	40	80	320[c]	40	40	160[c]	40	40	40
8 (F)	10	80[b]	640[c]	20	80[b]	640[c]	5	20	80[c]	10	20	80[c]	5	20	80[c]	5	20	80[c]
9 (I)	10	160[b]	640[c]	5	80[b]	320[c]	5	40[b]	320[c]	5	5	20	10	20	80[c]	20	40	640[c]
10 (F)	20	40	1280[c]	20	20	1280[c]	10	20	320[c]	5	10	320[c]	20	20	160[c]	20	40	640[c]
11 (F)	40	40	1280[c]	20	20	640[c]	20	20	640[c]	10	20	640[c]	20	20	160[c]	20	40	320[c]
12 (I)	40	40	1280[c]	5	20	1280[c]	5	40	1280[c]	5	10	640[c]	80	80	1280[c]	5	40	640[c]
13 (I)	40	40	320[c]	160	160	320	40	80	320[c]	20	20	160[c]	80	80	320[c]	80	80	640[c]
14 (F)	40	160[b]	640[c]	5	40[b]	1280[c]	40	40	320[c]	20	20	320[c]	40	40	320[c]	20	40	320[c]
15 (F)	40	320[b]	320	10	160[b]	320	40	160[b]	160	20	20	80[c]	40	40	160[c]	80	320[b]	40
16 (F)	80	160	640[c]	160	160	320	20	40	80	10	10	80[c]	20	20	80[c]	40	40	320[c]
17 (I)	80	160	640[c]	160	160	320	80	80	320[c]	40	40	160[c]	80	80	320[c]	80	80	320[c]

[a] *F* fluad, *I* intanza

[b] Seroconversion 1 month after vaccination

[c] Serologically diagnosed influenza virus infections based on seroconversion comparing HI titers in sera collected 1 and 6 months after vaccination

Table 2 Antibody response against different A(H3N2) antigens [A/Perth/16/09 (2011–2012 vaccine antigen), A/Victoria/361/2011 (2012–2013 vaccine antigen) and four epidemic drifted strains (A/PG/06/2012, A/PG/20/2012, A/PG/44/2012, A/PG/50/2012)] in 17 elderly volunteers found to be infected with A(H3N2) influenza virus after 2011–2012 influenza vaccination

	% seroprotected (≥40)		% seroprotected (≥160)		GMT		GMTR	% seroconversion
	[95 % C.I.]		[95 % C.I.]		[95 % C.I.]		[95 % C.I.]	[95 % C.I.]
Antigens (Assay)	Pre-vacc.	1 mo	Pre-vacc.	1 mo	Pre-vacc.	1 mo	1 mo/Pre-vacc.	Pre-vacc. vs 1 mo
A/Perth/16/2009	41.2	88.2**	0	35.3	16.3	67.9*	4.2	52.9
(HI)	[22.3–63.0]	[67.0–96.5]	[0.0–16.9]	[18.0–57.6]	[7.8–34.1]	[30.2–153.1]	[1.7–10.4]	[31.9–73.0]
A/Victoria/361/2011	17.6	70.6**	11.8	23.5	13.3	47.1**	3.5	47.0
(HI)	[6.5–39.7]	[48.0–86.2]	[3.5–33.0]	[10.0–46.0]	[5.8–30.3]	[24.1–91.8]	[1.5–8.4]	[27.0–68.1]
A/PG/06/2012	29.4	64.7*	0	5.9	15.0	34.0**	2.3	23.5
(HI)	[13.8–52.0]	[42.4–82.0]	[0.0–16.9]	[1.1–25.6]	[7.8–29.0]	[20.4–56.6]	[1.4–3.7]	[10.0–46.0]
A/PG/20/2012	11.8	17.6[a,b]	0	0	9.6	15.6[a,b]	1.6[b]	5.9[a,b]
(HI)	[3.5–33.0]	[6.5–39.7]	[0.0–16.9]	[0.0–16.9]	[5.6–16.4]	[8.6–28.7]	[1.0–2.7]	[1.1–25.6]
A/PG/44/2012	35.3	47.0[a]	0	0	17.6	32.6*[,a]	1.8[b]	11.8[a,b]
(HI)	[18.0–57.6]	[27.0–68.1]	[0.0–16.9]	[0.0–16.9]	[8.4–37.4]	[21.4–50.0]	[1.0–3.4]	[3.5–33.0]
A/PG/50/2012	35.3	70.6*	0	11.8	19.2	43.4	2.3[b]	17.6[a]
(HI)	[18.0–57.6]	[48.0–86.2]	[0.0–16.9]	[3.5–33.0]	[9.4–39.1]	[25.0–75.2]	[1.2–4.4]	[6.5–39.7]
A/Perth/16/2009	41.2	94.1**	5.9	35.3	19.2	94.2*[,c]	4.9	58.8
(Nt)	[22.3–63.0]	[74.4–98.8]	[1.1–25.6]	[18.0–57.6]	[8.5–43.2]	[52.0–170.6]	[2.0–11.9]	[37.0–77.6]
A/Victoria/361/2011	23.5	64.7*	0	11.8	11.3	37.7*	3.3	35.3
(Nt)	[10.0–46.0]	[42.4–82.0]	[0.0–16.9]	[3.5–33.0]	[5.7–22.4]	[16.6–86.0]	[1.4–3.7]	[18.0–57.6]

*$p < 0.05$; **$p < 0.01$ comparing pre-vaccination and 1 month after vaccination data
[a]$p < 0.05$; A:$p < 0.01$ comparing drifted strains with A/Perth/16/09 (2011–2012 vaccine antigen) (HI test)
[b]$p < 0.05$; B:$p < 0.01$ comparing drifted strains with A/Victoria/361/2011 (2012–2013 vaccine antigen) (HI test)
[c]$p < 0.01$ comparing A/Perth/16/2009 with A/Victoria/361/2011 (HI vs. HI and Nt vs. Nt)

neutralize the virus via entry/replication inhibition in mammalian cells possibly directed against the HA globular head and the HA stem region (Trombetta et al. 2014). To test the neutralizing capability of the antibodies evidenced with the HI test the sera of the infected volunteers were analyzed for Nt antibody titers against A/Perth/16/2009 and A/Victoria/361/2011 vaccine antigens, using a conventional neutralization test based on the inhibition of cytopathic effect (CPE) in MDCK cell cultures. Sera were inactivated at 56 °C for 30 min, serially diluted and incubated with 100 $TCID_{50}$/well before MDCK infection. After 3 days, the results were evaluated by detection of CPE and hemagglutination activity in the culture medium. The Nt titers were determined as the reciprocal of the last dilution neutralizing the viral replication. Although, there is no known protective level for neutralizing antibodies and regulatory authorities generally rely on the HI assay for commercialization of influenza vaccines, the results obtained for Nt titers, reported in Table 2, were examined according the same EMA criteria used for HI titers. No statistically significant differences were observed in pre-vaccination sera, although the values against the A/Victoria strain tended to be lower. One month after vaccination the three EMA criteria were fulfilled against the two vaccine antigens, however, the values of GMT Nt antibody against the new 2012–2013 A/Victoria/361/2011 vaccine antigen were significantly lower as compared with those found against the A/Perth/16/2009.

The relationship between HI and Nt titers was analyzed by Pearson's correlation test. A significant correlation, although at different levels, was found between the two tests examining titers found in pre- and 1 and 6 months post-vaccination sera both against A/Perth/16/2009 ($r = 0.88$; $r = 0.79$ and $r = 0.62$) and A/Victoria/361/2011 antigens ($r = 0.72$; $r = 0.65$ and $r = 0.85$).

Finally, the possibility of differences between the two groups of vaccinated people, one found to be infected after vaccination and the other not infected, was evaluated considering the demographic and health status characteristics and the immune response induced against the 2011–2012

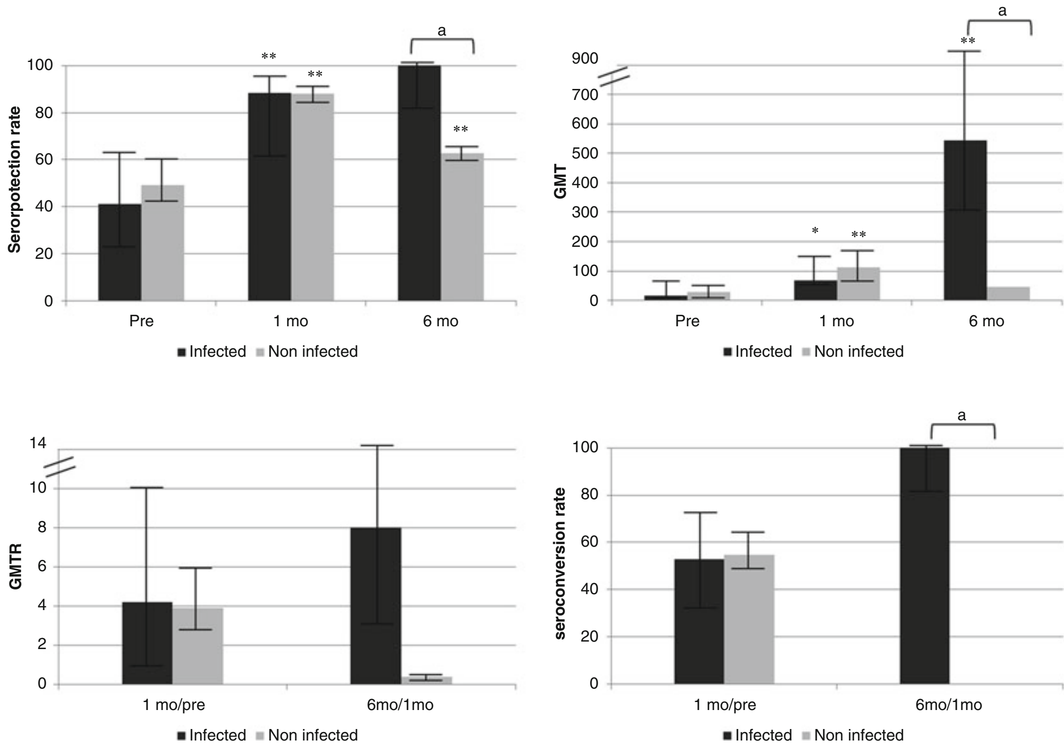
Table 3 Baseline characteristics of the 92 subjects participating in the study subdivided in two groups, influenza A(H3N2) infected and non-infected after 2011–2012 influenza vaccination

Baseline characteristics	Infected (N.17)	Non infected (N.75)
Mean age ± SD	85.8 ± 7.8	85.7 ± 7.1
(Range)	(69–99)	(68–101)
Good health (%)	0	1.6
Chronic use of drugs (%)[a]	100	91.9
Underlying diseases (%)[b]		
Cardiovascular disease	53.3	35.5
Diabetes	20.0	16.1
Respiratory disease	6.7	0
Cancer	6.7	4.8
Depression	33.3	22.6
Dementia	11.3	24.2

[a]Drugs most frequently used were antihypertensive/inotropic drugs and benzodiazepines
[b]Data available for 15 of the 17 infected volunteers and for 62 of the 75 non-infected volunteers; it was possible for each subject to have more than one disease

and 2012–2013 A(H3N2) vaccine antigens following 2011–2012 vaccine administration. As reported in Table 3, no statistically significant differences between the baseline demographic and clinical characteristics of the two groups were found. The HI titers found before, 1 and 6 months after vaccination in the two groups are reported in Fig. 1. Vaccination induced similar HI antibody titers in the two groups since no differences were found between infected and non-infected volunteers examining HI titers 1 month after vaccination. In the group of non-infected people, 6 months after vaccination, HI titers were lower as compared with those found 1 month after vaccination and very similar to the pre-vaccination values suggesting that waning of immunity might have facilitated infection of the 17 people.

In conclusion the results of this study showed serological evidence of recent influenza A (H3N2) infections in 17 elderly institutionalized people after vaccination with seasonal 2011–2012 influenza vaccine, in spite of the induction, 1 month post-vaccination, of positive HI and Nt antibody responses satisfying the



*:p<0.05; **:p<0.01 comparing pre-and 1 month or 1 and 6 months post=vaccination data

a: p<0.05 comparing infected and non-infected volunteers

Fig. 1 Comparison of HI antibody titers against 2011–2012 A(H3N2) vaccine component (A/Perth/16/2009) before, 1 and 6 months after vaccination in volunteers infected or non-infected after 2011–2012 vaccine administration. *: $p < 0.05$; **: $p < 0.01$ comparing pre- and 1 month or 1 and 6 months post-vaccination data. *a*: $p < 0.05$ comparing infected and non-infected volunteers

EMA criteria both against the A(H3N2) 2011–2012 and against the new drifted A (H3N2) 2012–2013 vaccine antigens. HI and Nt titers against the drifted A(H3N2) 2012–2013 vaccine antigen tended to be lower as compared with those found against the 2011–2012 vaccine antigen, although the differences, probably because of the low number of volunteer examined, were prevalently not statistically significant. Moreover, the results obtained suggest the circulation of antigenically different A(H3N2) influenza virus strains during the 2011–2012 winter season since examining immunological responses induced by vaccination against 4 drifted circulating A(H3N2) viruses we found a positive response only against 2 of these viruses (Tables 1 and 2). Viruses similar to the strains against which a positive response was not found might have been the first responsible of most of the infections observed in the vaccinated people. In addition, although we did not examine infection associated illnesses, our data evidenced, in accordance with other previous results (Masurel and Laufer 1984), that HI titers ≥40 might be not considered as an absolute titer directly correlated with protection from infection and that HI titer ≥160 are more protective at least in the older people and against A(H3N2) subtypes.

The comparison of demographic and immunological response after vaccination did not support the presence of differences between the group of vaccinated people infected after vaccination and the group of vaccinated people not infected (Table 3), and these results, even though we

could not examine other parameters, seem to suggest that infection of the 17 people could be due to differences in the exposure to influenza viruses between the two groups and that the infections might have been facilitated by the waning of vaccine induced immune response. Indeed the decline in the antibody levels against A(H3N2) viruses was evidenced by us in the group of non-infected people and is in accordance with previous data evidencing a significant rapid reduction of antibody levels following seasonal influenza vaccination especially in the elderly (Brydak et al. 2003; Reber et al. 2012; Song et al. 2010; Skowronski et al. 2008; Cate et al. 1983; Castilla et al. 2013; Jiménez-Jorge et al. 2013).

Although the limited number of volunteers examined did not allow to evaluate possible differences between the two vaccines used or between infections associated or not with ILI, our data confirm the reduced ability of seasonal influenza vaccines presently available in preventing influenza virus infection when drifted viruses are circulating.

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Advances in Microbiology, Infectious Diseases and Public Health: Fungal Occurrence in the Hair and Skin of Symptomatic Pets in Turin, Italy

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Abstract

Companion animals, often asymptomatic *reservoir* of fungi, can be important sources of infection in humans, due to the close contact with their owners. The present study was aimed to assess the occurrence of dermatophytes and other fungi isolated from pet dermatological lesions in Turin, Italy. Dermatological specimens were examined for fungal elements by direct microscopy and cultured to detect dermatophytes, other filamentous fungi and yeasts: 247 pets (118 cats, 111 dogs and 18 dwarf rabbits) were positive for fungal detection in culture. *Microsporum canis* was the most frequent dermatophyte in cats and dogs, whereas *Trichophyton mentagrophytes* was the most common in rabbits. Among the other fungi, for all examined pets, dematiaceous fungi were the most isolated, followed by *Mucorales*, penicilli, yeasts and yeast-like fungi, and aspergilli. No gender predisposition was detected for dermatophyte growth; on the contrary, for the other fungi male cats were more susceptible than female. The highest fungal occurrence was recorded in <1-year-old cats for dermatophytes, and in <5-year-old cats and dogs for the other fungi. Autumn was the period associated with a relevant incidence of fungal infection. Finally, fungi were more frequent in non pure-breed cats and in pure-breed dogs. These data underline the importance to timely inform

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pet owners about the potential health risk of infection caused not only by dermatophytes but also by non-dermatophyte fungi, routinely considered to be contaminants or harmless colonizers, since their role as source of zoonotic infections is not to be excluded.

Keywords
Dermatophytes • Non-dermatophyte fungi • Pets • Hair and skin lesions

1 Introduction

Considering the close contact between pets and their owners, especially between children and cats and dogs, these animals, often asymptomatic carries of dermatophytes, can be important sources of infection and/or carriers of infection (Mattei et al. 2014). In addition, evidence exists that rodents, such as rabbits, may be a risk of infection for their owners and for those who work closely with them (Torres-Rodríguez et al. 1992; Hata et al. 2000; Spiewak and Szostak 2000). It is widely known that animals are the *reservoir* of many dermatophytes belonging to the genera *Microsporum* spp. and *Trichophyton* spp., and that dermatophytoses are usually disseminated among domestic animals. *M. canis*, *M. gypseum* and *T. mentagrophytes* are the main etiological agents of clinical dermatophytosis in pets (Bond 2010; Kraemer et al. 2012). The disease is characterized by alopecia, scaling and crusting; however, other filamentous fungi could mimic dermatophyte lesions rendering them indistinguishable from that of dermatophytes. These non-dermatophytic fungi isolated from animal lesions could have pathogenic potential and/or keratinolytic activity. In fact many of these species, such as *Alternaria* spp., *Scopulariopsis* spp., *Penicillium* spp., *Rhizopus* spp. and *Fusarium* spp., are reported to be involved in fungal disease development and are increasingly recognized as agent of diseases both in animals and humans (Aho 1983; Bagy and Abdel-Mallek 1991; Seyedmousavi et al. 2015). Therefore, the aim of this report was to determine the occurrence, in Turin (Italy), of dermatophyte and non-dermatophyte fungi from living indoor cats, dogs and dwarf rabbits with lesions, referable to mycoses, for health monitoring since they are out by an appropriate health check.

2 Animals and Methods

2.1 Animals

In the period between March 2007 and November 2014, clinical dermatological specimens from 362 indoor domestic animals (195 cats, 149 dogs and 18 dwarf rabbits) were collected at Veterinary Clinics located in Turin. Pets, with suspected dermatophytosis, presented dermatological clinical signs such as scales, folliculitis, crusts and alopecic areas with variable degrees of inflammation and itch. Specimens (hair, scaling, crusts and/or skin scraping) were taken from head, abdomen, back and legs using a sterile lancet or pliers. The samples were submitted to the Bacteriology and Mycology Laboratory, Department of Public Health and Pediatrics, University of Torino, Turin, and processed.

2.2 Epidemiological Data Collection

The age, sex, breed, habitat in which animals lived and the presence of clinical signs were recorded for each animal. To assess the seasonal pattern of fungal infections, the sampling period was divided into four groups: spring (March–-May), summer (June–August), autumn (September–November) and winter (December–February).

2.3 Fungal Isolation and Identification

Specimens were examined for fungal elements by direct microscopy at 400× magnification after imbibitions in 20 % KOH. Multiple *inocula* (at least five) of the clinical specimens were cultured on Mycosel agar (MYC; Merck, Germany) to detect dermatophytes and Sabouraud dextrose agar (SAB; Sigma, St. Louis, Mo) for other filamentous fungi and yeasts. If the lesions were treated with antimycotics or covered in pus or other materials, they were first carefully washed with soap and water. The plates were incubated at 25 °C for at least 4 weeks and examined twice weekly. Cultures were held for at least 4 weeks before being considered negative. Each developing colony was isolated in pure culture on the following media: MYC (dermatophytes), Czapek's dox agar (Merck; aspergilli and penicillia), Potato dextrose agar (Merck; *Fusarium* spp.), modified Dixon agar (Merck; *Malassezia* spp.) and SAB (other filamentous fungi, yeasts and yeast-like fungi). The filamentous fungi, *Malassezia pachydermatis* and the yeast-like fungi were identified according to their colonial morphology and the microscopic appearance of the fungal elements (Raper and Fennell 1965; Rebell and Taplin 1979; Ellis 1993; Gueho et al. 1996; Guillot et al. 1996; de Hoog et al. 2000; Pitt 2000), whereas the yeasts were identified by API ID 32C (bioMérieux Italia S.p.A.; Italy).

2.4 Statistical Analysis

The chi-square test was performed for the analysis associations of the categorized variables: sex, age, season and breed. A p value of <0.05 was considered significant.

3 Results

This study included 362 symptomatic pets with marked skin lesions, characterized by alopecic areas, more or less itching, scabbed, disseminated in several body regions (head, abdomen, back, legs; data not shown), indistinguishable between dermatophytic and non-dermatophytic ones.

Out of 362 domestic animals, 282 were positive for fungal elements at direct examination and 247 were positive for fungal detection in culture (118 cats, 111 dogs and all 18 dwarf rabbits; Table 1). 54.25 % of cat samples, 38.75 % of dog samples and 27.78 % of rabbit samples were positive for dermatophytes: *M. canis* was the most frequent dermatophyte isolated from cats and dogs, whereas *M. gypseum* and *T. mentagrophytes* were isolated from 2 dogs and 5 rabbits, respectively.

The remaining fungal cultures (54.66 %; Table 1) were positive for other filamentous fungi and yeasts. In details: dematiaceous (*Alternaria alternata, Epicoccum nigrum*, *Cladosporium cladosporioides*, *C. sphaerospermum*, *C. herbarum*, *Aureobasidium pullulans* and *Nigrospora* spp.) for 34.44 %; hyaline mycetes, represented by penicilli (*Penicillium brevi-compactum, P. griseofulvum*, *P. waksmanii*), aspergilli (*Aspergillus niger*, *A. versicolor* and *A. fumigatus*), *Trichoderma harzianum*, *T. viride* and *Fusarium* spp. for 10.11 %; *Mucorales*, represented by *Rhizopus oryzae* and *Mucor hiemalis,* for 6.07 %; yeasts and yeast-like fungi, represented by *Candida* spp., *M. pachydermatis* and *Geotrichum candidum*, for 4.04 %.

In all positive animals, males were more than females (Table 2); however no gender predisposition was detected for dermatophyte growth; on the contrary, male cats were significantly ($p = 0.0224$) more susceptible than female for other fungi. It can be highlighted the highest dermatophyte occurrence in <1-year-old cats ($p < 0.0001$) and the presence of other fungi in <5-year-old positive cats ($p < 0.0001$) and dogs ($p = 0.0276$; Table 2). All positive rabbits were less than 1-year-old. Positive samples for dermatophytes and other fungi were recorded in autumn (September–November) for all companion animals: a significant seasonal difference was detected for dogs ($p = 0.0168$; Table 2). Finally, fungi were more frequent in pure-breed dogs and in non pure-breed cats (Table 2), without statistical significant differences.

Table 1 Isolation and occurrence of fungal species (%)

	Cats		Dogs		Rabbits		Total	
	118/195[a]		111/149		18/18		247/362	
	(60.51 %)		(74.50 %)		(100 %)		(68.23 %)	
	Positive animals examined							
	n	%	n	%	n	%	n	%
Dermatophytes								
Microsporum canis	64	54.25	41	36.95	–	–	105	42.51
M. gypseum	–	–	2	1.80	–	–	2	0.81
Trichophyton mentagrophytes	–	–	–	–	5	27.78	5	2.02
Total	**64**	**54.25**	**43**	**38.75**	**5**	**27.78**	**112**	**45.34**
Dematiaceous mycetes								
Alternaria alternata	16	13.56	18	16.22	–	–	34	13.78
Epicoccum nigrum	11	9.32	14	12.61	–	–	25	10.12
Cladosporium cladosporioides	5	4.24	7	6.31	–	–	12	4.87
C. sphaerospermum	2	1.69	2	1.80	–	–	4	1.62
C. herbarum	–	–	2	1.80	–	–	2	0.81
Aureobasidium pullulans	–	–	2	1.80	4	22.22	6	2.43
Nigrospora spp.	2	1.69	–	–	–	–	2	0.81
Total	**36**	**30.50**	**45**	**40.54**	**4**	**22.22**	**85**	**34.44**
Hyaline mycetes								
Penicillium brevi-compactum	5	4.24	2	1.80	4	22.22	11	4.46
P. griseofulvum	1	0.85	–	–	–	–	1	0.40
P. waksmanii	–	–	2	1.80	–	–	2	0.81
Aspergillus niger	2	1.69	–	–	–	–	2	0.81
A. versicolor	–	–	1	0.90	–	–	1	0.40
A. fumigatus	–	–	4	3.61	–	–	4	1.62
Trichoderma harzianum	1	0.85	–	–	–	–	1	0.40
T. viride	1	0.85	–	–	–	–	1	0.40
Fusarium spp.	–	–	2	1.80	–	–	2	0.81
Total	**10**	**8.48**	**11**	**9.91**	**4**	**22.22**	**25**	**10.11**
Zygomycetes								
Rhizopus oryzae	3	2.54	5	4.50	5	27.78	13	5.26
Mucor hiemalis	2	1.69	–	–	–	–	2	0.81
Total	**5**	**4.23**	**5**	**4.50**	**5**	**27.78**	**15**	**6.07**
Yeasts and yeast-like fungi								
Candida tropicalis	1	0.85	–	–	–	–	1	0.40
C. albicans	–	–	2	1.80	–	–	2	0.81
Malassezia pachydermatis	2	1.69	3	2.70	–	–	5	2.02
Geotrichum candidum	–	–	2	1.80	–	–	2	0.81
Total	**3**	**2.54**	**7**	**6.30**	**–**	**–**	**10**	**4.04**

[a]Positive/total; n = number of cases of isolation; % = percentage frequency of occurrence (calculated per number of positive animals sampled)

4 Discussion

Over the past two decades, studies of dermatophytoses from domestic or wild animals have been described worldwide (Brilhante et al. 2003; Khosravi and Mahmoudi 2003; Cafarchia et al. 2004; Bond 2010; Kraemer et al. 2012). In some countries, such as Italy and France, *M. canis* is the most common etiological agent, whereas in Spain it varies in relation to the geographical area (Torres-Rodríguez

Table 2 Prevalence of dermatophytes and other fungi in cats, dogs and rabbits in relation to epidemiological variables[a]

		Cats				Dogs				Rabbits			
		Dermatophytes		Other fungi		Dermatophytes		Other fungi		Dermatophytes		Other fungi	
		Positivity/n	%	Positivity/n	%	Positivity/n	%	Positivity/n	%	Positivity/n	%	Positivity/n	%
Sex	Male	34/121	28.10	39/121	32.23	24/85	28.23	39/85	45.88	–	–	13/13	100
	Female	30/74	40.54	15/74	20.27	19/64	29.69	29/64	45.31	5/5	100	–	–
		$\boldsymbol{p = 0.0224}$				$p = 0.7867$				$\boldsymbol{p < 0.0001}$			
Age	<1 year	41/96	42.71	17/96	17.71	22/62	35.48	24/62	38.71	5/18	27.78	13/18	72.22
	1–5 years	16/81	19.75	33/81	40.74	9/45	20.0	25/45	55.55	–	–	–	–
	>5 years	7/18	38.89	4/18	22.22	12/42	28.57	19/42	45.24	–	–	–	–
		$\boldsymbol{p < 0.0001}$				$\boldsymbol{p = 0.0276}$				N.A.			
Seasons	Spring	14/38	36.84	9/38	23.68	4/21	19.04	12/21	57.14	–	–	4/4	100
	Summer	4/15	26.67	5/15	33.33	7/23	30.43	10/23	43.48	–	–	–	–
	Autumn	32/101	31.68	29/101	28.71	22/78	28.21	36/78	46.15	5/5	100	–	–
	Winter	14/41	34.15	11/41	26.83	10/27	37.04	10/27	37.04	–	–	9/9	100
		$p = 0.3695$				$\boldsymbol{p = 0.0168}$				N.A.			
Breed	Cross-breed	–	–	–	–	15/39	38.46	14/39	35.90	–	–	–	–
	Pure-breed	23/59	38.98	13/59	22.03	28/110	25.45	54/110	49.09	5/18	27.78	13/18	72.22
	Other breed	41/136	30.15	41/136	30.15	–	–	–	–	–	–	–	–
		$p = 0.1216$				$p = 0.1216$				N.A.			

[a]The chi-square test was used for the analysis associations of the categorized variables: sex, age, season and breed
A p value of <0.05 was considered significant

et al. 1992). In our study (Table 1) *M. canis* was the most frequent dermatophyte isolated in cats and dogs, confirming previous reports in Turin and in other sites in Italy, indicating that this fungus did not vary over the years (Marchisio et al. 1995; Mantovani 1978; Chermette et al. 2008; Bond 2010); *M. gypseum* and *T. mentagrophytes* were isolated from dogs and rabbits, respectively, underlying that these dermatophytes affect other pets (Chermette et al. 2008; Bond 2010). Additionally, our data report 5 *M. canis* isolated from asymptomatic cats (data not shown) whose owners manifested skin mycoses, indicating that cats are at present recognized as major sources of infection for their owners, confirming literature data (Cafarchia et al. 2006). As reported by Bond (2010), asymptomatic carriers cats are especially risky for humans, because no precautions are taken to prevent potential transfer; however, such cats may progress to develop overt infection and more abundant arthroconidia shedding. Infected cats have been shown to cause substantial environmental contamination and a significant airborne load of viable fungal elements, whereas dogs are of lesser importance in this regard.

Other filamentous fungi are common in the environment and their conidia are transported by air currents and settled on pet fur. Among these moulds, dematiaceous fungi and *Fusarium* spp., isolated in this study (Table 1), are nowadays well recognized as etiological agents of mycosis in animals and humans too (Bagy and Abdel-Mallek 1991; Noble et al. 1997; Huttova et al. 1998; Kluger et al. 2004; Walsh et al. 2004; Sanchez and Larsen 2007; Fan et al. 2009; Ryoo et al. 2009). For example, a case of *Alternaria* peritonitis after contact with a cat and the involvement in pet skin infections of *Fusarium* spp., a well-recognized cause of human diseases, were reported (Kluger et al. 2004; Ryoo et al. 2009). In this study *Alternaria*, *Epicoccum*, *Cladosporium* and *Fusarium* isolates probably played a role in the pathogenicity: they were no sporadic and many colonies were seen on the plates in each case.

Furthermore, we isolated some saprophytic fungi, commonly found in air and soil, such as *Mucorales* besides penicilli and aspergilli (Table 1). Albeit the recovery of these fungi was consistent with the findings of other authors (Bagy and Abdel-Mallek 1991; Keller et al. 2000; Efuntoye and Fashanu 2002; Ledbetter et al. 2007), further studies are required to verify and confirm their pathogenesis in companion animals.

Trichoderma spp., a saprophytic fungus commonly found in soil, isolated only from a cat in our study, has been reported among emerging fungal pathogens for both animals and humans (Table 1) (Kluger et al. 2004; Kantarcioğlu et al. 2009).

From a veterinary point of view, our findings related to the yeast *M. pachydermatis* from cat and dog skin lesions may have a great significance (Table 1). It can be found in very large proportion on the skin of healthy animals and it is the only lipid-independent species in the genus *Malassezia*; however since the early 1990s *M. pachydermatis* was isolated from lesions of atopic dermatitis, flea allergic dermatitis, otitis externa, pyoderma and seborrheic dermatitidis in dogs and cats (Aizawa et al. 2001; Dorogi 2002; Khosravi et al. 2010). Although *M. pachydermatis* is not normally isolated from human skin, there have been several reports of *M. pachydermatis*-associated fungaemia in infants in neonatal intensive care unit and in adults with serious internal diseases (Bond et al. 2010; ESCCAP Guideline 2011).

Literature data on sex, age, seasonality and breed are still controversial (Khosravi and Mahmoudi 2003; Cafarchia et al. 2004; Cabanes et al. 1997). With regard to the sex, from our results, in both cats and dogs no significant difference between the sexes for dermatophyte growth has been detected. Among cats, males were significantly more susceptible than females to other fungi occurrence (Table 2): this may be accounted for a different composition of sebum between males and females, as suggested by Cafarchia et al. (2004). For age, our data show that young animals are more susceptible to fungal infections (Table 2). Adult animals tend to be more resistant to infections than young animals in relation to their changes in the skin and

secretions (quantity and nature of sebaceous lipids in the epidermis), hair replacement cycle, and development of an immune response to keratinophylic moulds (Bond 2010; Cafarchia et al. 2004; Rotstein et al. 1999; Khosravi and Mahmoudi 2003). Although the risk of dermatophyte infection is greater for puppies, kittens and aged or debilitated animals, the infection is not strictly age or health status-related, and so the risk continues throughout life. Consideration should be given to provide all dogs and cats with appropriate dermatophyte control throughout their lives (ESCCAP Guideline 2011). From our study autumn was the period with the highest risk for fungal infection (Table 2), according to Mancianti et al. (2002) and Iorio et al. (2007). The prevalence of non-dermatophyte and dermatophyte filamentous fungi varies according to the climate, temperature, relative humidity and rainfall of different geographical regions or natural *reservoir* (Brilhante et al. 2003; Cabanes et al. 1997; Mancianti et al. 2002; Iorio et al. 2007). Moreover, the life style such as the tendency to live in the outdoor environment in contact with soil, in groups, in isolation or in proximity to humans; the hygiene; the differences in non-specific cutaneous defenses are the general conditions related to the higher prevalence of fungal infections (de Hoog et al. 2000; Brilhante et al. 2003; Cafarchia et al. 2006). In our study in both cats and dogs there was difference in fungal isolation related to breed since fungi were more frequent in non pure-breed cats and in pure-breed dogs (Table 2). Actually, breed is not proved to be a predisposing factor for infection (Cafarchia et al. 2006; Mancianti et al. 2002).

"The disease is not clear, unless we seek it": contact with animals or contaminated environments represents the major risk of infection for humans and people in contact with infected animals should be advised of the risk. In fact, nowadays, lack of connection between the monitoring of diseases in animals and humans is still great. The best way to bypass infection is to prevent the contact: this prophylactic strategy is very simple but not always feasible because infected animals do not show obvious clinical signs. When lesions are evident, the dermatophyte clinical lesion appearance is often indistinguishable from that caused by other fungi, suggesting the need for greater and accurate control, monitoring and identification of these last species to avoid the overestimated clinical diagnosis of dermatophytoses and to address the appropriate therapy. The role of animals as source of zoonoses in dermatophyte is widely accepted; on the contrary further investigations to evaluate the considerable zoonotic and zoopathogenic potential of other fungi, routinely considered to be contaminants or harmless colonizers, are necessary. A better understanding of diseases in pets could have direct relevance for the prevention and the fight against infectious diseases of humans.

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Infectious Agents Associated with Head and Neck Carcinomas

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Abstract

In addition to traditional risk factors such as smoking habits and alcohol consumption, certain microbes also play an important role in the generation of head and neck carcinomas. Infection with high-risk human papillomavirus types is strongly associated with the development of oropharyngeal carcinoma, and Epstein-Barr virus appears to be indispensable for the development of non-keratinizing squamous cell carcinoma of the nasopharynx. Other viruses including torque teno virus and hepatitis C virus may act as co-carcinogens, increasing the risk of malignant transformation. A shift in the composition of the oral microbiome was associated with the development of oral squamous cell carcinoma, although the causal or casual role of oral bacteria remains to be clarified. Conversion of ethanol to acetaldehyde, a mutagenic compound, by members of the oral microflora as well as by fungi including *Candida albicans* and others is a potential mechanism that may increase oral cancer risk. In addition, distinct *Candida* spp. also produce NBMA (N-nitrosobenzylmethylamine), a potent carcinogen. Inflammatory processes elicited by microbes may also facilitate tumorigenesis in the head and neck region.

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Keywords
Human papillomavirus • Oropharyngeal carcinoma Epstein-Barr virus • Nasopharyngeal carcinoma • Co-carcinogen • Torque teno virus • Hepatitis C virus • Oral microbiome • Mutagenic compound • *Candida albicans*

1 Introduction

Head and neck cancer is a broad term that encompasses cancers arising in the head and neck region. They may originate from the mucosal lining of the oral cavity, nasal cavity, paranasal sinuses, oropharynx, nasopharynx, hypopharynx, and larynx, or begin in the lip or in the salivary glands (Bose et al. 2013). Most of these malignancies are head and neck squamous cell carcinomas (HNSCCs). Head and neck cancer is the sixth most common cancer type worldwide with approximately 650 000 new cases annually (Ferlay et al. 2010). Despite the advancements of treatment methods, including chemotherapy, radiotherapy, and surgery, the 5-year survival rate of HNSCC patients improved only modestly in the past decades: it is around 50 %, mostly due to locoregional recurrences, distant metastases and additional primary tumors (Leemans et al. 2011).

The major risk factors for HNSCC are tobacco use and alcohol consumption and they seem to have a multiplicative combined effect. Genetic polymorphisms in enzymes that metabolize tobacco and alcohol have been linked to an increased risk for HNSCC (Cadoni et al. 2012; Maurya et al. 2014). Smokeless tobacco and chewing of betel quid are also known risk factors for oral cancer (Li et al. 2015; Sand et al. 2014). There is wide geographic variation in the incidence and anatomic distribution of HNSCC worldwide. This variation is predominately attributed to demographic differences in the habits of tobacco use and alcohol consumption. In the western part of the world the incidence of HNSCC has declined, mostly due to the decline of tobacco use. In contrast, oral cancer is the leading type of malignancies among men in high risk countries, such as India, Pakistan, Sri Lanka and Bangladesh, (Joshi et al. 2014). Similarly, the estimated incidence and mortality rate of lip, oral cavity and pharynx carcinomas is high in Central and Eastern European countries (Hungary, Slovakia, Romania), possibly due to traditional risk factors (Iriti and Varoni 2015).

According to the International Agency on Research for Cancer (IARC), in 2008 around two million of the estimated 12.7 million new cancer cases occurring worldwide could be attributed to infections (IARC Working Group 2012; de Martel et al. 2012). In addition to oncogenic viruses (HPV, MCPyV, EBV, HHV-8, HBV, HCV, HTLV-I) and bacteria (*Helicobacter pylori*) other infectious agents may also contribute to the development of malignant tumors. A series of microbe-induced pathological alterations including mutations, cell cycle modulation, inhibition of DNA repair, epigenetic dysregulation, inflammation and immune system impairment may facilitate tumorigenesis (Alibek et al. 2013).

One of the well-documented virus-cancer relationships is the association of high-risk human papillomavirus (HPV) infection with a subset of HNSCCs located predominantly to the oropharynx. In the US and Western Europe, there was a recent increase in oropharyngeal cancer incidence, compared to other head and neck cancers. This phenomenon was attributed to a higher prevalence of high-risk HPV strains in the oral mucosa (Mehanna et al. 2013; Näsman et al. 2009; Rietbergen et al. 2013).

The nearly ubiquitous Epstein-Barr virus (EBV) plays a major role in the development of a series of neoplasms including undifferentiated nasopharyngeal carcinoma (NPC). NPC is a rare cancer globally, but it is the leading cancer type in distinct high-risk populations, especially in Southern China, indicating that non-viral, genetic and environmental factors also contribute to NPC development (Jia and Qin 2012). Although compared to HPV and EBV the evidence is less direct, recent data also suggested a role for other viral, as well as bacterial and fungal infections in the etiology of head and neck cancer (Sand and Jalouli 2014).

Carcinogenesis is a multistep process driven by genetic and epigenetic alterations that result typically in the clonal or oligoclonal expansion of cells (Hanahan and Weinberg 2000). Over 90 % of HNSCCs arise from pre-existing potentially malignant lesions or conditions, e.g. oral leukoplakia and oral lichen planus. The treatment of high risk oral premalignancies, however, did not efficiently prevent either their recurrence or the development of oral carcinomas (Braakhuis et al. 2003; Leemans et al. 2011). This phenomenon was attributed to the existence of a "field effect", i.e. genetic alterations predisposing large areas of oral mucosa for tumorigenesis ('field cancerization'). Recurrences and novel new malignant transformations occur preferentially at such 'fields' (Braakhuis et al. 2003; Leemans et al. 2011). It is worthy to note that epigenetic abnormalities characteristic for laryngeal squamous cell carcinomas may also extend to the adjacent normal mucosa, indicating the occurrence of an "epigenetic field of cancerisation" (Paluszczak et al. 2011).

We wish to overview the contribution of infectious agents including viruses, bacteria and fungi to the development of HNSCC. A better understanding of microbe-induced molecular events, including genetic and epigenetic changes, in head and neck cancer development may pave the way for novel therapies and prevention strategies.

2 Human Papillomavirus

Human papillomaviruses (HPVs) of the *Papillomaviridae* family are small, non-enveloped viruses with a double-stranded, circular DNA genome of about 8000 bp. There are 174 completely characterized HPV types, classified on the basis of the capsid protein L1 gene sequences (Bzhalava et al. 2013). The discovery that distinct HPV types were associated with cervical carcinoma was a significant milestone in tumor virology (reviewed by zur Hausen 2009).

HPVs have strict host selectivity for humans. Cutaneous types infect the skin, whereas mucosal types infect nonkeratinized squamous epithelia lining the oral cavity, the esophagus and the vagina. HPVs can be grouped into high and low risk types based on their capacity to induce malignant transformation. High risk types include HPV 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58,59, 66, 68, 73 and 82; they were associated with high grade intraepithelial lesions of the cervix and with invasive cancer. In contrast, non-oncogenic or low-risk HPV types, including HPV 6, 11, 40, 42, 43, 44, 54, 61, 72, 81 and 89, were detected in low grade intraepithelial lesions (Woods et al. 2014).

HPV is associated with wide range of diseases from benign warts to invasive cancer. There is strong epidemiological evidence for the involvement of HPV infection in the generation of six non-skin cancer types, including the carcinomas of cervix, penis, vulva, vagina, anus and upper aerodigestive tract (de Martel et al. 2012). The majority of HPV related head and neck cancers are located to the oropharynx, an anatomic region comprised of the soft palate, uvula, tonsils, posterior pharyngeal wall, and the base of the tongue (Gillison et al. 2014). It was suggested that tonsillar crypts may trap the virus and inhibit mechanical clearance. In addition, the monolayer of epithelial cells that lines these crypts could be more susceptible to HPV infection than stratified epithelium (Elrefaey et al. 2014; Klussmann et al. 2001). It is worthy to note that the oropharyngeal SCCs associated with traditional risk factors, i.e. smoking and alcohol consumption, are usually moderately differentiated and show a keratinizing phenotype. In contrast, the majority of HPV-associated head and neck cancers lack significant keratinization and are of basaloid morphology (Westra 2009).

3 The HPV Genome

All open reading frames in the 8 kb HPV genome are located on one of the two DNA strands. Most HPV-infected cells carry circular, episomal HPV DNA molecules, whereas HPV-associated carcinomas harbor viral genomes integrated into the cellular DNA. The HPV genome can be divided into three major regions. Early gene transcription and replication is regulated by the long control region (LCR) that contains promoter and enhancer elements as well as the viral origin of replication. The downstream coding ORFs are

called early and late genes and their names refer to their location as well as the timing of their transcription. The products of the early genes (E1-E7) are necessary for the genome maintenance and replication. E5, E6 and E7 are the oncoproteins of HPV, although they have their important role in the normal lifecycle of the virus as well. The E2 protein, apart from having a role in genome replication, regulates the transcription of the other early genes. The late genes (L1 and L2) code for the structural proteins, with L1 being the major and L2 the minor capsid protein (Rautava and Syrjänen 2012; Stanley 2012).

4 HPV Epidemiology

As for the prevalence of HPV in oropharyngeal cancer cases, there are significant differences between various anatomical regions. A recent meta-analysis by Mehanna et al. found 47.7 % overall pooled HPV prevalence in oropharyngeal cancer, whereas 21.8 % of non-oropharyngeal squamous cell carcinomas were HPV positive. HPV prevalence was found to significantly increase over time from 40,5 % before 2000 to 72,2 % after 2005. The overall HPV prevalence of head and neck malignancies differed by geographical region as well: North-America and Europe had the highest prevalence. In the last decades Northern and Western European countries have reported a steep rise in the proportion of HPV associated oropharyngeal cancers (Mehanna et al. 2013; Marur et al. 2010; Näsman et al. 2009; Rietbergen et al. 2013; Louie et al. 2015). However, regions with low prevalence of HPV have reported lower prevalence of HPV DNA in HNSCC patients (López et al. 2014), and similarly low prevalence was found in relatively high-risk regions for HNSCC, in Central-Europe and Latin-America. In these communities traditional risk factors may play a more important role in the development of HNSCCs (Ribeiro et al. 2011; Marur et al. 2010).

HPV can be found in other types of head and neck cancers as well. Isayeva et al. analyzed the prevalence rates of HPV in oral cavity, laryngeal, sinonasal and nasopharyngeal carcinomas. Lower prevalence rates were detected (20.2 %, 23.6 %, 29.6 % and 31.1 %, respectively) compared to oropharyngeal cancers (Isayeva et al. 2012). They also found that the prevalence of HPV in potentially premalignant and premalignant oral lesions is significantly higher than the rate of oral HPV carriers. Interestingly, submucous fibrosis showed the highest HPV prevalence (11/12, 91.7 %). Submucous fibrosis is a potentially malignant condition of the oral cavity and it is linked to chewing of betel quid (Jalouli et al. 2010).

Independently of overall HPV prevalence in HNSCCs, HPV16 was the most abundant HPV type found in these malignancies. HPV16 accounted for 86.7 % of HPV positive oropharyngeal cancer; it was less prevalent, however, in other types of head and neck cancer. A smaller proportion was attributable to HPV18; other high risk types were rarely found in these cancers (Kreimer et al. 2005; Chaturvedi 2012). It was observed that HPV associated oropharyngeal cancers tend to have better prognosis than HPV-positive carcinomas located to other anatomic regions (Lindquist et al. 2007; Ang et al. 2010; El-Mofty 2012, Mellin et al. 2012, Sethi et al. 2012; Ramqvist et al. 2015).

5 Characteristics of Patients with HPV-Associated Head and Neck Cancer

People diagnosed with HPV related HNSCC tend to be younger (<60 years of age) than those with HNSCC caused by traditional risk factors (>60 years of age) (Marur et al. 2010; Genden et al. 2013). Although the absolute proportion of HNSCC affecting young adults (18–40 years of age) remains low (1–6 %), epidemiological studies have shown a steady rise in the incidence of oropharyngeal and oral cavity cancer in this population (Majchrzak et al. 2014).

Several aspects of sexual behavior are strongly associated with HPV positive HNSCC. These include younger age at first intercourse, total lifetime number of vaginal and oral sex partners and lack of barrier use during sexual intercourses (Gillison et al. 2008; Chaturvedi 2012; Burke et al. 2014).

The incidence of both HPV-positive and HPV-negative HNSCC are higher in men. The male to female ratio has declined in oral cancer, and now it is about 1.5:1; however in HPV-associated HNSCC the male:female ratio remains 3:1 (Gillison et al. 2012; Warnakulasuriya 2009). This phenomenon can't be explained exclusively by the differences in sexual behavior between the two genders, and suggests some male predisposition to oropharyngeal cancer. It might be attributed to a protective effect of seroconversion in women due to earlier cervical HPV infection (Safaeian et al. 2010), or to the higher oral HPV prevalence among men. The latter is possibly due to a more effective transmission of HPV through oral sex on women versus men (Gillison et al. 2012).

6 HPV Induced Carcinogenesis: Integration of the Viral Genome into the Host Cell DNA

After initial infection, the HPV genome persists in episomal form within the host cells. Typically, there is no integration of the viral genome into the cellular DNA during productive infection of differentiating epithelial cells. The virus relies on the cellular replication machinery for the replication of its own genome (Lazarczyk et al. 2009). Malignant transformation is associated with high-level expression of viral E6 and E7 oncoproteins. During HPV-initiated carcinogenesis, high-level E6 and E7 expression frequently occurs after the integration of the viral DNA into the host cell genome (Vinokurova et al. 2008). The integration breakpoint is usually within the E2 gene encoding a negative regulator of E6 and E7 transcription. Thus, the integration of the viral genome disrupts the regulatory function of E2 and leads to constitutively active high-level E6 and E7 expression and increased cell proliferation (Parfenov et al. 2014; Williams et al. 2011; Rautava and Syrjänen 2012). The integration event also causes genomic instability by inducing chromosomal rearrangements, DNA amplification and disruption of tumor suppressor genes (Parfenov et al. 2014). In certain neoplasms, however, the HPV DNA can also be found in episomal form, or there is a combination of episomal and integrated form. In HPV16-associated oropharyngeal cancer the episomal or combined forms of viral genomes dominate. In cases when HPV remained in episomal form, a higher episomal count and a high viral load was detected (Mellin et al. 2002; Deng et al. 2013; Parfenov et al. 2014; Olthof et al. 2015).

7 HPV Oncoproteins

Three of the HPV proteins encoded by the early region of the viral genome, E5, E6 and E7 were implicated in carcinogenesis. The E5 gene is frequently lost during integration and a large fraction of HPV-associated tumors do not express E5 protein (reviewed by Venuti et al. 2011). E5 might contribute to oncogenesis, however, in case of HPV genomes that persist as episomes in tumor cells (Venuti et al. 2011). E5 may play a role in immune evasion by reducing the MHC-I level on the cell surface (Stöppler et al. 1996; Campo et al. 2010). In addition, E5 may promote cell growth by enhancing epidermal growth factor receptor-mediated signaling (DiMaio and Mattoon 2001).

In high-risk HPV-associated oropharyngeal carcinoma cells, continuous expression of the E6 and E7 oncoproteins is essential for the maintenance of the transformed phenotype (Rampias et al. 2009). The mechanisms contributing to E6 and E7 mediated oncogenesis are complex. Here we wish to outline only some of the best documented carcinogenetic pathways.

E7 has neither direct DNA binding activity nor enzymatic activity, but it is able to interact with key cellular regulators. E7 binds and induces the degradation of the retinoblastoma protein (Rb), a tumor suppressor protein that regulates the G1-S transition of the cell cycle (reviewed by Rautava and Syrjänen 2012; Boyer et al. 1996). Rb interacts with the E2F family of transcription factors that activate genes indispensable for S-phase entry and progression (McLaughlin-Drubin and Münger 2010). Thus, binding of E7 to Rb results in constitutive expression of E2F responsive genes and

leads to DNA synthesis. Degradation of pRB induces the upregulation of p16^{INK4A} (p16) tumor suppressor protein, and the elevated p16 level is used as a diagnostic marker in HPV positive HNSCC (Fakhry et al. 2014). E7, a pleiotropic regulator, also interacts with a series of other cellular proteins involved in the control of cell cycle and affects the gene expression pattern of host cells by binding to key epigenetic regulators (reviewed by Klingelhutz and Roman 2012; Moody and Laimins 2010).

The E6 protein of high-risk HPVs forms complex with, and targets the tumor suppressor protein p53 for proteasomal degradation by recruiting the cellular ubiquitin ligase E6AP. In addition, the E6-p53 interaction interferes with the binding of p53 to DNA and blocks p53 acetylation (McLaughlin-Drubin and Münger 2010). Because HPV-associated oropharyngeal cancers harbor wild type, non-mutated p53 that has a pro-apoptotic activity, it was suggested that disrupting the E6-p53 complex may induce apoptosis in HPV related malignancies (Li and Johnson 2013; Caicedo-Granados et al. 2014).

Genomic instability is an early event in HPV-associated carcinogenesis (Moody and Laimins 2010). In addition to the integration of the HPV genome into the host cell DNA, HPV E6 and E7 may also contribute to the development of chromosomal aberrations, partly by blocking p53, an important factor maintaining the stability of the genome, and partly by inducing centrosome abnormalities (McLaughlin-Drubin and Münger 2010).

It is worthy to note that HPV directly inhibits interferon synthesis and signaling *via* the interaction of E6 and E7 proteins with components of the interferon signaling pathways (reviewed by Stanley 2012). Such a mechanism may facilitate the immune escape of HPV-positive carcinomas.

8 Epstein-Barr Virus: The First Human Tumor Virus

Epstein-Barr virus (EBV), a human gammaherpesvirus, is associated with both lymphomas and carcinomas, including Burkitt's lymphoma (BL), Hodgkin's lymphoma, midline granuloma, post-transplant lymphoproliferative disorders (PTLDs), X-linked lymphoproliferative syndrome, nasopharyngeal carcinoma (NPC), gastric carcinoma, and others (reviewed by Shah and Young 2009; Sugden 2014). In immunosuppressed and immunodeficient patients, EBV-related leiomyosarcomas, i.e. smooth muscle neoplasms may also develop (Dalal et al. 2008). EBV, the first human tumor virus, was discovered in BL cell cultures. Although it was initially considered a purely lymphotropic virus, an *in situ* hybridization study of anaplastic NPCs revealed the presence EBV DNA in the carcinoma cells, but not in infiltrating lymphocytes, suggesting a role for EBV in the malignant transformation of epithelial cells, too (Epstein et al. 1964; Sugden 2014; Wolf et al. 1973).

9 Estein-Barr Virus: Basic Facts

Epstein-Barr virus (EBV, also known as human herpesvirus 4, HHV-4) belongs to the genus *Lymphocryptovirus* within the subfamily *Gammaherpesvirinae* of the family *Herpesviridae*. The prototype EBV genome is 172 kbp in length and it is packaged into the virions as a linear DNA molecule. Upon infection of B-lymphoid and epithelial cells, the linear EBV genome undergoes circularization, and latent EBV genomes typically persist as circular episomes attached to the nuclear matrix. Latent EBV episomes co-replicate with the cellular DNA and display a restricted gene expression pattern. In contrast, all viral genes are expressed and a large number of linear EBV genomes are generated upon induction of lytic, productive EBV replication. Depending on the host cell phenotype, latent EBV genomes adopt distinct gene expression patterns (latency types) and the activity of latent viral promoters is regulated by the cellular epigenetic machinery (reviewed by Takacs et al. 2010). In turn, latent, growth-transformation associated EBV proteins affect the host cell transcriptome and epigenome through the interaction with cellular epigenetic regulators (reviewed by Niller et al. 2009).

10 EBV and Nasopharymgeal Carcinoma: Epidemiology

EBV is a ubiquitous herpesvirus spreading among humans most commonly through saliva and other bodily fluids. The majority of the population undergoes inapparent primary infection in early childhood. The virus replicates in oropharyngeal epithelial cells, but also infects B lymphocytes, and latent EBV genomes are carried by resting, memory B cells for life. Primary EBV infection in teenagers or adults causes infectious mononucleosis (IM, also called glandular fever), a self-limiting disease (reviewed by Niller et al. 2007; Sugden 2014).

Nasopharyngeal carcinoma was the first cancer of the head and neck region that was found to be associated with a human virus (Wolf et al. 1973).

There are two major types or classes of nasopharyngeal carcinoma, keratinizing squamous cell carcinoma accounting for 20 % of NPC cases, and non-keratinizing squamous cell carcinoma. Keratinizing squamous cell carcinomas of the nasopharynx occur sporadically throughout the world at a relatively low incidence. They are either EBV-positive or EBV negative, depending on the geographical area (Nicholls et al. 1997). The non-keratinizing type represents 80 % of NPC cases and it is invariably associated with EBV (reviewed by Shah and Young 2009). Although EBV infects human populations all over the world, the incidence of the non-keratinizing type of NPC, including both differentiated and undifferentiated forms carrying latent EBV episomes in almost 100 % of cases, occurs with a high incidence at restricted geographical locations, i.e. it is an endemic tumor in Southeast Asia, Tunisia, and among Alaskan and Greenland Inuit (reviewed by Shah and Young 2009; Niller et al. 2007).

11 Risk Factors Affecting the Incidence of Nasopharyngeal Carcinoma

Several genetic risk factors were identified in China, especially in the Guangzhou area, that may contribute to the high NPC incidence. These include an allele encoding a member of cytochrome-P450 super-family of proteins involved in the activation of carcinogenic compounds, a gene coding for a glutathione S-transferase that contributes to the detoxification of carcinogens, as well as genes located to or near to HLA loci and genes encoding enzymes and regulators of the DNA repair machinery (reviewed by Lung et al. 2014; Niller et al. 2007). It is worthy to note that epigenetic inactivation of cellular genes, mainly by promoter hypermethylation, also plays an important role in the generation of NPC (reviewed by Niller et al. 2014; Li et al. 2011).

Additional risk factors are volatile nitrosamines that may act as initiators of chemical carcinogenesis and phorbol ester-like compounds that may act as promoters, stimulating the proliferation of cells carrying mutated, initiated genomes. Such compounds are present in medical herbal teas or in the diet, including salted fish. Phorbol-ester-like compounds such as diterpene-esters may also induce lytic EBV replication, i.e. reactivation of latent EBV genomes in B cells infiltrating the nasopharyngeal epithelium (Ito et al. 1983; Ho et al. 1978). Increased local EBV load may facilitate the infection of epithelial cells, an early event in NPC development.

12 NPC: Carcinogenesis

As briefly outlined above, in addition to EBV infection, genetic factors and environmental carcinogens also play a role and act in concert during the initiation and progression of NPC. Based on these observations, Lo et al. elaborated a collaborative model for NPC tumorigenesis (Lo et al. 2012). They suggested that DNA damage elicited by carcinogens (e.g. nitrosamines from salted fish and preserved food) and local chronic inflammation might cause chromosomal aberrations that could be detected in the dysplastic lesions of the nasopharynx. A typical finding was the deletion of the short arm of chromosome 3 (3p) affecting several tumor suppressor genes. In addition, epigenetic mechanisms such as promoter hypermethylation also contribute to the silencing of tumor suppressor genes already in

EBV-negative dysplastic lesions. Centrosome abnormalities and the generation of multipolar spindles may also induce genetic instability at this stage. Loss of the *p16* tumor suppressor gene may result in the overexpression of cyclin D1 that favours stable latent EBV infection of nasopharyngeal epithelial cells. EBV infection and the clonal proliferation induced by latent EBV proteins and RNAs may accelerate neoplastic development (Lo et al. 2012).

Viral gene expression patterns of NPC typically corresponds to the so called latency type II.: in addition to the nuclear protein EBNA1 (EBV nuclear antigen 1), a variable expression of LMP1 (latent membrane protein 1) and LMP2A (latent membrane protein2A) is observed in NPC (reviewed by Niller et al. 2007). LMP1 is an oncoprotein, its expression in rodent cells results in malignant transformation. The EBV genome encodes non-translated microRNAs as well that act as posttranscriptional regulators of mRNA and protein levels. Hsu et al. observed that miR-BART9 targets the E-cadherin mRNA and promotes migration and metastasis formation by NPC cells (Hsu et al. 2014).

13 Other Viruses Possibly Associated with Head and Neck Carcinomas and Oral Precancerous Lesions: Torque Teno Virus (TTV) and Hepatitis C Virus (HCV)

Torque Teno viruses (TTVs) belong to the family of *Anelloviridae* and have a small, circular, single stranded DNA genome (reviewed by Spandole et al. 2015). Although the first TTV-like sequence was found in the serum of a patient with post-transfusion hepatitis, at present, TTV is not linked to either hepatitis or any other disease as a causative agent (Nishizawa et al. 1997; Okamoto 2009). TTVs are ubiquitous viruses with a nearly 100 % prevalence that establish persistent infection (Saback et al. 1999; Hsieh et al. 1999; Zhong et al. 2001; Ninomiya et al. 2008; Hussain et al. 2012; Vasilyev et al. 2009). Children may be infected by the end of their first year and simultaneous infections may also occur (Ninomiya et al. 2008). TTVs can be found in wide range of tissues and body fluids including liver, bone marrow, lymph nodes, spleen, pancreas, thyroid, lungs, kidneys, PMBCs, saliva, urine, tears, nasal secretion, feces, throat swabs, bile and semen (Spandole et al. 2015). In addition, TTV related sequences were detected in many different human diseases including AIDS, neoplasia, asthma and rheumatoid arthritis (Moen et al. 2002; Thom and Petrik 2007; Pifferi et al. 2005; Saláková et al. 2009; Figueiredo et al. 2007; Suzuki et al. 2014; de Villiers et al. 2007; Gergely et al. 2006). Regarding head and neck cancer, TTV related sequences were found in laryngeal cancer (de Villiers et al. 2002). Furthermore, co-infection with genogroup 1 TTV and HPV was associated with poor clinical outcome of laryngeal carcinoma and the co-prevalence of these viruses was significantly higher in lesions of oral squamous cell cancer and oral lichen planus compared to healthy mucosa (Szládek et al. 2005; Fehér et al. 2009). TTV DNA activates Toll-like receptor 9 (TLR9) and induces the production of different pro-inflammatory cytokines. Thus, TTV may affect the severity of diseases where inflammation plays an important role (Rocchi et al. 2009; Maggi and Bendinelli 2009). In addition, TTVs encode a microRNA (TTV-tth8 miRNA) that interferes with interferon signaling (Kincaid et al. 2013). TTV-tth8 miRNA may play a role in immune evasion by TTV and by TTV infected cells by interacting with the mRNA of a regulatory protein, N-myc (and STAT) interactor (NMI) that modulates interferon and cytokine signaling (Kincaid et al. 2013).

Hepatitis C virus (HCV), an enveloped, positive-sense single stranded RNA virus is a member of the *Flaviviridae* family; HCV causes chronic liver disease (Mohd Hanafiah et al. 2013). HCV infection is also associated with several extrahepatic manifestations (EHMs) (Zignego et al. 2007). One of the EHMs is oral lichen planus (OLP). It is worthy to note, however, that the association of HCV infection and OLP was stronger in Mediterranean countries and in Japan, whereas in northern Europe OLP was not associated with chronic liver disease caused by HCV (Carrozzo 2008). OLP is a chronic inflammatory disease that affects the skin and also the oral mucosa. The malignant transformation rate of

Table 1 Viruses associated with head and neck carcinoma

Virus	Major viral oncoproteins	Neoplasm or precancerous lesion
Human papillomavirus	E6, E7	Oropharyngeal carcinoma, non-keratinizing; basaloid phenotype
Epstein-Barr virus	LMP1, LMP2A, EBNA1 (?)	Nasopharyngeal carcinoma, non-keratinizing type
Torque teno virus	?	Laryngeal carcinoma (?)
Hepatitis C virus	?	Oral lichen planus (?)

OLP was found to be quite low (1.09 %); still, it may play a role in the development of oral cancer (Fitzpatrick et al. 2014).

Further studies may clarify the role of TTV and HCV in HNSCC development.

Table 1 summarizes the viruses associated with head and neck carcinomas.

14 Bacteria Associated with Head and Neck Carcinoma

Although tobacco smoking, alcohol intake and HPV16 infection appear to be major, independent risk factors of a HNSCC, especially oral carcinoma, a series of observations indicate that oral bacteria and fungal infections of the oral cavity may also be associated, either casually or causally, with oral neoplasia. In a pioneering study, Nagy et al. analysed the biofilm flora present on the surfaces of oral squamous cell carcinomas and on the contiguous healthy mucosa (Nagy et al. 1998). They found a higher number of both aerobic and anaerobic colony forming units at the tumour sites than at the apparently healthy mucosa. They also compared the distribution of aerobic and anaerobic bacterial species at these anatomical sites. The frequency of most aerobic species was similar at both sites, except *Serratia liquefaciens*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *beta-hemolyzing Streptococci*, and *Enterococcus faecalis* that were isolated more frequently from the biofilm samples obtained from the surfaces of oral carcinomas. Regarding anaerobic species, the frequency of peptostreptococci and lactobacilli was comparable at both sites, whereas *Actinomyces* spp., *Propionibacterium* spp., *Clostridium* spp., *Veilonella* spp., *Fusobacterium* spp., *Prevotella* spp., *Porphyromonas* spp., and *Bacteriodes ureolyticus*/*gracilis* was isolated more frequently from the tumor surface than from the control mucosal surface (Nagy et al. 1998). It is worthy to note that the fungus *Candida albicans* was detected in a significant fraction of oral carcinomas, but not at control sites (Nagy et al. 1998). Nagy et al. concluded that the cancer lesion itself may predispose patients with oral carcinoma to both local and systemic infections (Nagy et al. 1998). In a follow-up study, they demonstrated that topical antimicrobial treatment of oral squamous cell carcinoma lesions effectively reduced the number of biofilm-associated bacteria (Nagy et al. 2000). It is worthy to note that radiotherapy or cytostatic treatment of HNSCC patients also affected the composition of oral microbiota, resulting in an increased risk of local and systemic infections by pathogenic or opportunistic microbes (reviewed by Meurman 2010).

Hooper et al. aimed at the localization of bacteria within a surface-decontaminated oral squamous cell carcinoma (OSCC) sample; they performed *in situ* hybridization with a FITC-labeled oligonucleotide recognizing a sequence within the 16S rRNA gene of *Bacteria* (Hooper et al. 2007). They found bacteria throughout the tumor tissue. Analysis of bacterial species by PCR cloning and sequencing of the 16S rRNA gene revealed that there was a trend for an enrichment of *Clavibacter michiganensis*, *Fusobacterium naviforme*, *Ralstonia insidiosa* and *Prevotella* spp. in the tumor-derived samples whereas control tissue samples were enriched in *Granulicatella adiacens*, *Porphyromonas*

gingivalis, *Sphingomonas* spp. and *Streptococcus mitis/oralis* (Hooper et al. 2007). Hooper et al. speculated the acidic and hypoxic microenvironment may select for the growth of certain bacterial species within tumors. They also raised the point that tumor-associated bacteria may play a role in carcinogenesis (Hooper et al. 2007). Others also emphasized that in addition to *Helicobacter pylori,* which is the causative agent of gastric carcinoma and gastric lymphoma in humans, other bacterial species could also be involved in tumorigenesis (Lax and Thomas 2002).

Pushalkar et al. used denaturing gradient gel electrophoresis and 16S rRNA gene sequencing to compare the oral microbiota of OSCC patients (Pushalkar et al. 2012). There were no significant differences in phylogenies at tumor and non-tumor sites, although four *Streptococcus* species as well as *Peptostreptococcus stomatis*, *Gemella haemolysans*, *Gemella morbillorum*, and *Johnsonella ignova* were highly associated with the tumor site. At the non-tumor site *Granulicatella adiacens* was prevalent. Pushalkar et al. noticed site- specific and subject-specific differences in the distribution of bacterial species. They suggested that certain oral bacteria may associate with different stages of OSCC and may contribute to the acidic and hypoxic milieu characteristic for neoplasms (Pushalkar et al. 2012).

Schmidt et al. performed pyrosequencing and also next generation sequencing (using the Illumina MiSeq instrument) to reveal the diversity of microbiomes in samples obtained by swabbing of oral cancer lesions and clinically normal mucosal surfaces (Schmidt et al. 2014). Based on the analysis of the V4 region of the bacterial 16S rRNA genes, they classified 65,037 sequences at the genus level and 17,115 sequences at the species level. They observed a reduced abundance of the phyla *Firmicutes* (especially *Streptococcus*) and *Actinobacteria* (especially *Rothia*) in cancer and pre-cancer samples compared to the anatomically matched clinically normal patient samples. In contrast, the proportion of *Fusobacteria* increased at the tumor site. Although there were inter-individual differences, these changes appeared to be consistent. Schmidt et al. argued that in spite of the diversity of the oral microbial community, only distinct, biofilm-forming oral bacteria adhere to oral tissues, followed by secondary colonizers. They suggested that altered surface properties at OSCC lesions may affect the adherence of bacteria, and a shift in bacterial populations may induce inflammatory responses favouring tumor progression (Schmidt et al. 2014). It is worthy to note that *Fusobacterium nucleatum*, a Gram-negative oral bacterium capable to invade the oral mucosa, was recently implicated in colon carcinogenesis (Castellarin et al. 2012; Kostic et al. 2012, 2013).

Bebek et al. amplified, cloned and sequenced variable regions 1–4 of the prokaryotic 16S rRNA gene to characterize bacterial populations in paired HNSCC and normal mucosa samples (Bebek et al. 2012). They also analysed the DNA methylation pattern of four cellular promoters (*MDR1*, *IL8*, *RARB*, *TGFBR2*) directing the expression of genes implicated in inflammation and tumorigenesis. Interestingly, hypermethylation of the *MDR1* promoter, a phenomenon regularly associated with promoter silencing, correlated with the presence of bacteria belonging to the *Enterobacteriaceae* family and the *Tenericutes* phylum (Bebek et al. 2012). *MDR1* codes for multidrug resistance protein 1, a drug efflux pump for xenobiotic compounds and MDR1 hypermethylation may contribute to the progression of gastric carcinoma (Tahara et al. 2009). Bebek et al. speculated that inflammatory processes elicited by bacteria may facilitate tumorigenesis by inducing hypermethylation of distinct cellular promoters (Bebek et al. 2012). It is worthy to note that the phylum *Tenericutes* includes the genera *Mycoplasma* and *Ureaplasma* which are prevalent in oral samples from STD patients (Nakashima et al. 2014). Others identified *Mycoplasma salivarium* as a dominant colonizer of oral carcinoma in two Fanconi anaemia patients, and it was also observed that *Mycoplasma fermentans* and *Mycoplasma penetrans* induced malignant cell transformation *in vitro* (Henrich et al. 2014; Tsai et al. 1995; Feng et al. 1999; Zhang et al. 1997).

The distribution of bacterial species detected in the saliva may reflect the microbial diversity of the soft tissues located in the oral cavity. Thus, in principle, the salivary microbiota could be used as a diagnostic marker of OSCC. Mager et al. observed that the salivary counts of *Capnocytophaga gingivalis*, *Prevotella melaninogenica*, and *Streptococcus mitis* were elevated in the saliva of OSCC patients compared to the unstimulated saliva samples of OSCC-free subjects (Mager et al. 2005). Mager et al. speculated that alterations of tumor cell receptors may facilitate the adherence of certain bacteria and the resulting shift of soft tissue microbiota in the oral cavity may affect the levels of bacteria in the saliva (Mager et al. 2005). A recent study also found a shift in the saliva microbiome of OSCC patients: the most prevalent genera were *Streptococcus*, *Gemella*, *Rothia*, *Peptostreptococcus*, *Lactobacillus*, and *Porphyromonas*. In contrast, in control saliva samples *Prevotella*, *Neisseria*, *Leptotrichia*, *Capnocytophaga*, *Actinobacillus*, and *Oribacterium* dominated (Pushalkar et al. 2011).

Although the exact role of oral bacteria in carcinogenesis remains to be clarified, one potential mechanism is the generation of carcinogenic metabolites by certain oral bacteria (Meurman and Uittamo 2008). It was demonstrated that both non-pathogenic *Neisseria* strains and strains of *Streptococcus salivarius* and *Streptococcus intermedius* as well as *Corynebacterium* spp. and *Stomatococcus* spp. are capable to convert ethanol to acetaldehyde, a mutagenic and carcinogenic substance (Muto et al. 2000; Homann et al. 2000; Kurkivuori et al. 2007). Such a mechanism may explain how poor dental status associated with bacterial overgrowth may increase oral cancer risk in patients with tooth loss, poor dentition and inadequate oral hygiene (Homann et al. 2000, 2001). Homann et al. demonstrated an increase in salivary acetaldehyde production from ethanol in saliva samples of patients with poor dental status (Homann et al. 2001).

Table 2 summarizes the bacteria associated with head and neck carcinomas and their putative role in carcinogenesis.

15 Fungi Associated with Head and Neck Carcinoma

In parallel to the alterations of the bacterial flora in oral cancer (see above), Nagy et al. described the presence of *Candida albicans* in a significant fraction of oral carcinomas, but not at control sites (Nagy et al. 1998). Furthermore, similarly to certain oral bacteria, it was documented that both *Candida albicans* strains and non-Candida albicans yeasts were capable of salivary acetaldehyde production from ethanol, and *Candida albicans* could frequently be detected in oral epithelial dysplasia, a premalignant lesion with an increased risk of oral cancer (Tillonen et al. 1999; McCullough et al. 2002; Nieminen et al. 2009, reviewed by Sitheeque and Samaranayake 2003; Bakri et al. 2010).

Chronic hyperplastic candidiasis (CHC, also referred to as candidal leukoplakia) is a clinical term for *Candida*-infected oral leukoplakias of the oral mucosa that are characterized by hyphal invasion and parakeratinosis. Although most frequently *Candida albicans* could be detected in CHC lesions, other species including *Candida dubliniensis*, *Candida tropicalis*, *Candida pintolopesii*, *Candida glabrata* and *Sacharomyces cerevisiae* were also detected in adherent chronic white patches of the oral mucosa (Cernea et al. 1965; Jepsen and Winther 1965; Krogh et al. 1986, reviewed by Bakri et al. 2010). *C. albicans* may either colonize existing premalignant or malignant oral lesions, or may promote the generation of precancerous conditions and their progression to cancer (Cernea et al. 1965; Jepsen and Winther 1965; Nagy et al. 1998; Sitheeque and Samaranayake 2003; Bakri et al. 2010; Sanjaya et al. 2011). A strong argument for a carcinogenic role of *Candida* infection is the production of carcinogens by certain *Candida spp.*: in addition to the mutagenic acetaldehyde (see above), formation of the potent carcinogen N-nitrosobenzylmethylamine (NBMA) was also observed (Krogh et al. 1987; Krogh 1990). Production of proteinases and pro-inflammatory mediators by *Candida* spp. may also contribute, indirectly, to carcinogenesis

Table 2 Bacteria associated with oral carcinomas and their putative role in carcinogenesis or tumor progression

Bacterium	Putative role	Reference
Serratia liquefaciens	?	Nagy et al. (1998)
Klebsiella pneumoniae	?	
Citrobacter freundii	?	
beta-hemolyzing Streptococci	?	
Enterococcus faecalis	?	
Actinomyces spp.	?	
Propionibacterium spp.	?	
Clostridium spp.	?	
Veilonella spp.	?	
Fusobacterium spp.	?	
Prevotella spp.	?	
Porphyromonas spp.	?	
Bacteriodes ureolyticus/ gracilis	?	
Clavibacter michiganensis	?	Hooper et al. (2007)
Fusobacterium naviforme	?	
Ralstonia insidiosa	?	
Prevotella spp.	?	
Streptococcus spp.	Contribution to the acidic and hypoxic milieu	Pushalkar et al. (2012)
Peptostreptococcus stomatis		
Gemella haemolysans, *Gemella morbillorum*		
Johnsonella ignova		
Fusobacterium spp.	?	Schmidt et al. (2014)
Enterobacteriaceae (family)	Induction of cellular promoter hypermethylation; induction of pro-inflammatory changes	Bebek et al. (2012)
Tenericutes (phylum)		
Streptococcus salivarius	Conversion of ethanol to mutagenic acetaldehyde	Muto et al. (2000), Homann et al. (2000), Kurkivuori et al. (2007)
Streptococcus intermedius		
Corynebacterium spp.		
Stomatococcus spp.		

Table 3 Fungi associated with oral carcinomas and their putative role in carcinogenesis

Fungus	Putative role	References
Candida spp.	Salivary acetaldehyde production from ethanol	Tillonen et al. (1999)
	Formation of carcinogen (nitrosobenzylmethylamine, NBMA)	Krogh et al. (1987), Krogh (1990)

by degrading cell surface proteins, basement membrane and extracellular matrix components and by eliciting chronic inflammation (reviewed by Bakri et al. 2010). Table 3 summarizes the fungi associated with head and neck carcinomas and their putative role in carcinogenesis

16 Conclusions

In the head and neck region, besides traditional risk factors such as smoking habits and alcohol consumption, certain microbes also play a role in

the generation of malignant epithelial tumors. Infection with high-risk human papillomavirus types is strongly associated with the development of oropharyngeal carcinoma and Epstein-Barr virus appears to be indispensable for the development of non-keratinizing squamous cell carcinoma of the nasopharynx. Infection with other viruses including torque teno virus and hepatitis C virus may increase the risk of initiation or progression head and neck carcinomas. A shift in the composition of the oral microbiome was also associated with oral squamous cell carcinoma, although the exact role of oral bacteria remains to be clarified. Conversion of ethanol to acetaldehyde, a mutagenic compound, by members of the oral microflora as well as by fungi including *Candida albicans* and others may increase oral cancer risk. In addition, distinct *Candida spp*. also produce NBMA (N-nitrosobenzylmethylamine), a potent carcinogen. Inflammatory processes elicited by microbes may also facilitate tumorigenesis in the head and neck region.

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Imported and Indigenous cases of Invasive Meningocococcal Disease W:P1.5,2:F1-1: ST-11 in migrants' reception centers. Italy, June-November 2014

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Abstract

We report about three unliked cases of meningococcal meningitis caused by the ST-11/ET-37 strain of *Neisseria meningitidis* serogroup W. Two of the three cases, detected in Sicily on June and July 2014, were migrants from Mali and Eritrea. The third case was a fatal meningitis occurred on November 2014 in a 37 years old man, working in an immigrant center in Calabria. This report suggests that tetravalent conjugate vaccines (ACYW) should be actively offered to the staff of migrants' reception centers.

Keywords

Neisseria meningitidis • Serogroup W • Typing • Migrant

Invasive meningococcal disease (IMD) due to *Neisseria meningitidis* serogroup W is uncommon in European countries. Although this serogroup accounts only for a small proportion of IMD cases in Italy, an increase from 2.1 % in 2010 to 4.7 % in 2014 has been observed (http://www.iss.it/mabi/).

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Hereby, we report on three unlinked cases of IMD due to serogroup W, occurred between June and November 2014, among individuals attending or working in migrants reception centers located in the South of Italy.

The first case was an 18 years old man from Mali, who arrived to the southern coast of Sicily on June 2014, after crossing the Mediterranean sea. The man developed high fever (>38 °C) and other symptoms suggestive of meningitis, 7 days after his arrival (Stephens 1999). The second case was a 20 years old woman from Eritrea, who arrived in Lampedusa on July 2014. She developed symptoms suggestive of meningitis while resident in a camp and was admitted to the infectious disease clinic in Palermo. Both patients survived.

Chemoprophylaxis with rifampicin or ciprofloxacin was administered to all the individuals who had a direct exposure to the index cases during the period of time in which the cases were assumed to be infectious (i.e., from 7 days prior to the onset of symptoms to 24 h after the initiation of antibiotic prophylaxis) in order to reduce the risk of transmission.

On November 2014, in Calabria, a Region of the South of Italy, in a location where no cases of IMD had yet been reported among migrants, a fatal case of meningitis due to serogroup W occurred in a 37 years old man of Moroccan nationality, working as cultural mediator in an immigrants' reception center.

To our knowledge, this man did not have any exposure outside the workplace which might have placed him at risk of acquiring meningococcal infection due to an exotic strain, and he had not been outside Italy during the last year. Antibiotic prophylaxis was given to all his close contacts.

A lumbar puncture showed purulent cerebrospinal fluid (CSF) in all the three cases. DNA extraction from CSFs was performed by using commercial kits (Qiagen, Hilden, Germany), leading to the molecular identification of *N. meningitidis*. The Reference Laboratory of the Istituto Superiore di Sanità (ISS) in Rome, Italy, confirmed the diagnosis of serogroup W infection and performed the Multilocus sequence typing (MLST), and PorA and FetA typing, in accordance with the procedures published in http://neisseria.org/. The *N. meningitidis* W: P1.5,2:F1-1:ST-11 clonal complex (cc) strain was identified in all the samples.

The identification of three cases infected with the W/ST-11 cc strain associated with migrant reception camps is particularly interesting since, in the last years, most of endogenous IMD cases of W in Italy (73 %) were typed as ST-22 cc and only the remaining, as ST-11.

The three cases of IMD described above, caused by the same serogroup W strain, are paradigmatic of the changes occurring in meningococcal serogroup dynamics in a globalized world. The first two cases were represented by two migrants, who developed IMD several days after being arrived in a migrant reception facility in Sicily; both of them were from Sub-Saharan Africa, and one of them was from Mali, a country of the meningitis belt which is considered endemic for W/ST-11 cc strain (Guindo et al. 2011). Although, we cannot completely rule out that the infection was acquired during the travel, or even in the camp, it is likely that it was imported from the country of origin. To this regard, it has been suggested that the W/ST-11 cc Haji strain may cause infection up to more than 1 month post-return (Wilder-Smith et al. 2003). Concerning the third case, although IMD cases were not identified in the camp, the detection of the same clonal complex and the exclusion of risky exposures outside the camp strongly support an occupational risk due to contacts with carriers coming from endemic countries.

Meningococcal disease due to W was reported in Africa since the early 1980s (Kwara et al. 1998). In 1996, carriage of W strains was reported during a vaccine trial on Gambian children (MacLennan et al. 2000). Since the Hajj pilgrimage of the year 2000, serogroup W/ST-11 has emerged as a strain responsible of epidemic of IMD in Africa (Kelly and Pollard 2003; Parent du Chatelet et al. 2012). The first reported outbreak of W occurred in 2002 in Burkina Faso, with a large epidemic of about 8,000 cases (Nathan et al. 2007). In 2013, many countries, such as Benin, Burkina Faso, Ivory Coast, Gambia and Ghana, reported W/ST-11 cc as the

predominant strain and the main cause of outbreaks (World Health Organization 2013).

The increasing number of immigrants from African countries raises a series of issues regarding the introduction of serogroups which are rarely found in persons living in Western European countries; to this purpose, outbreaks due to the W:P1.5,2:F1-1:ST-11(cc11) strain have been documented in France and in the UK (Parent du Chatelet et al. 2012; Puleston et al. 2012).

The two imported cases reported in Sicily and the indigenous case detected in a migrant reception center represent an early warning signal for Italy. Moreover, because of unauthorized migration flows, many migrants are in irregular situations, lacking access to health services. Thus, there is a need to ensure access to early diagnosis and treatment for disadvantaged populations (Severoni and Dembech 2014).

Before drawing conclusions, some limits of the study need to be mentioned. Firstly, there is no definitive evidence that both migrants acquired meningococcal infection in their country of origin and not during their travel or even in the reception centers. Secondly, although the IMD affecting the cultural mediator was likely to be due to occupational exposure, any other contact outside the receptions camp could not be completely ruled out. Finally, carriage studies, which may be extremely useful in improving the knowledge of meningococcal transmission dynamics, were not conducted; unfortunately, they had not been planned, and the prompt administration of prophylaxis regimens was considered the priority in terms of public health intervention.

In conclusion, increased migration flows from endemic areas and the importation of exotic strains of *N. meningitidis* launches new challenges to Mediterranean Europe. The identification of cases among migrants and indigenous workers attending reception facilities suggests the importance of adopting more stringent public health measures, such as early detection of cases, improvement of surveillance systems, screening of migrants and staff for nasopharyngeal carriage, and active offer of tetravalent conjugate meningococcal vaccine (ACYW) to the staff of reception centers.

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Role of Sonication in the Microbiological Diagnosis of Implant-Associated Infections: Beyond the Orthopedic Prosthesis

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Abstract

Implant-associated infections are difficult-to-treat conditions associated with high morbidity, mortality and length of hospitalization. They are characterized by biofilm formation on implant surface, which makes the microbiological diagnosis difficult and requires a complete device removal for the correct management. The sonication method, which is based on the application of long-wave ultrasounds radiating in a liquid medium, has been recently validated for the diagnosis of prosthetic joint infections. Additionally, this technique has been considered a potential tool in order to improve the microbiological diagnosis of infections associated with other foreign bodies, such as breast, urinary, endovascular and cerebral implants. In the present study, the application of sonication in the setting of implant-associated infections other than orthopedics will be reviewed.

Keywords

Implant Associated Infections (IAIs) • Sonication • Microbiological diagnosis

1 Introduction

The rate of implant positioning has increased over time, mostly due to the rise of median age and the increased prevalence of cardiovascular, neurological and bone/joint diseases (Zhang et al. 2014; Bradshaw et al. 2014). In addition, the growing incidence of tumors has led to the need of breast reconstruction surgery, long-term central venous and urinary catheters use (Jung et al. 2015).

Although rare, implant-associated infections (IAIs) have been increasing worldwide and are associated with high morbidity and mortality.

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The diagnosis of IAIs may be a real challenge for physicians, due to the wide variety of presenting symptoms and to their chronic and relapsing nature (Trampuz and Zimmerli 2008; Baddour et al. 2010; Hasse et al. 2013). In order to obtain a microbiological diagnosis, the complete device removal is required; however, traditional cultures often give negative results because adherent bacteria that are encased in biofilms on the surface of implanted device can encumber microorganism detection (Stewart and Costerton 2001). In recent years, the development of ultrasounds-based technologies aimed at improving the microbiological diagnosis of IAIs has been investigated.

So far, many studies evaluated the role of sonication method in the setting of prosthetic joint infections (PJIs), leading to its validation in the microbiological diagnosis of these infections (Tunney et al. 1998; Trampuz and Zimmerli 2005; Trampuz et al. 2007). In fact, culture of samples obtained by prostheses sonication has found to be more sensitive than conventional periprosthetic-tissue cultures for the microbiological diagnosis of prosthetic hip and knee infections, especially in patients with previous antimicrobial therapy (Trampuz et al. 2007). In addition, sonicate fluid culture was more sensitive than periprosthetic tissue culture in the setting of prosthetic shoulder infections (Piper et al. 2009) and at least as sensitive as periprosthetic tissue culture to detect prosthetic elbow infections (Vergidis et al. 2011).

Among several advantages including the possibility of performing molecular (Achermann et al. 2010; Portillo et al. 2012) and immunological studies on sonication fluid, the quantification of the number of microorganisms and the detection of polymicrobial growth represent additional important tools whose knowledge might augment and spread the use of this method. In fact, a CFU cut-off in the sonication fluid has been established for distinguishing PJIs from aseptic failures (Trampuz et al. 2007) and for diagnosing Central Venous Catheters (CVC)-related infections (Mermel et al. 2009) whereas only preliminary data are found for external ventricul drains (EVD)/ventriculo-peritoneal shunts infections (VPS) (Jost et al. 2014) or for cardiac device infections (CDIs) (personal data, not shown).

In addition, the sonication method has shown the ability to isolate different bacterial phenotypes such as small colony variant (SCV) and multidrug resistant (MDR) bacteria. SCV, which is a slow-growing phenotype associated with intracellular persistence and fastidious growth requirement, has been recognized as a leading cause of IAIs including CDIs (Tumbarello et al. 2012a) and PJIs (Piffaut et al. 2013).

Furthermore, in an era of MDR bacteria, the microbiological diagnosis of IAIs is crucial for choosing the optimal antimicrobial treatment. In this setting, our group demonstrated that a MDR *Corynebacterium striatum* causing pacemaker lead endocarditis could have been detected only throughout sonication (Oliva et al. 2010).

On the other hand, a disadvantage of sonication is represented by the potential risk of contamination, which might occur during sample processing.

The role of sonication method in the microbiological diagnosis of IAIs other than PJIs is an area of active investigation. This technique has been considered a potential essential tool in order to improve the microbiological diagnosis of infections associated with other foreign bodies such as breast, urinary, endovascular and cerebral implants. In the present study, the application of sonication in the setting of implant-associated infections other than orthopedics will be reviewed.

2 Sonication Technique

Since it has been established in the late 1990s (Tunney et al. 1998), a technique based on the application of long-wave ultrasounds (defined by frequencies above the range of human hearing, 20 kHz) has been used in order to enhance bacterial growth by liberating sessile organisms embedded in biofilm on foreign bodies (Nguyen et al. 2002; Klug et al. 2003; Carmen et al. 2005; Bjerkan et al. 2009; Rieger et al. 2009; Sampedro et al. 2010; Bonkat et al. 2011).

Technically, ultrasound waves radiate through a liquid media and produce high- and

low-pressure areas. During the low-pressure phase, lots of microscopic bubbles form and then collapse during the high-pressure phase by releasing a high amount of energy on the surface of the foreign body. This agitation causes a vacuum-scrubbing action able to dislodge bacteria (Pitt and Ross 2003; Trampuz et al. 2003). The mechanism through which ultrasounds exert their activity on bacteria is the phenomenon of acoustic cavitation (Joyce et al. 2003), which is considered to influence both size and formation of cavitation bubbles.

Another application of this method is the lysis of bacterial cells. Whether bacteria are dislodged from foreign bodies or are lysed depends on several factors such as acoustic frequency, energy, temperature and time of ultrasound exposure. For instance, biofilm removal by sonication strongly depends on the intensity of sonication energy (power density) and, to a lesser extent, on frequency (Pitt 2005).

For low ultrasonic frequencies (20–40 kHz), large cavitation bubbles form and generate high energy when they collapse. However, at higher frequencies (580 kHz), the acoustic cycle is shorter with a minor time for cavitation bubble formation; therefore, the cavitation bubbles are smaller and collapse with low energy (Joyce et al. 2003).

The duration of sonication has been recognized as an important factor influencing the viability of bacteria. In fact, the more is the length of ultrasound exposure, the more is the probability that bacteria are killed. A previous study showed a significant reduction in live/viable bacterial cell numbers after 15 min treatment at low frequencies (Joyce et al. 2003).

Among different sonication protocols (Tande and Patel 2014), the most widely used for dislodging bacteria from foreign bodies are based on 1-min (Trampuz et al. 2007) or 5-min duration of sonication (McDowell and Patrick 2005; Sampedro et al. 2010; Oliva et al. 2013), with or without the centrifugation as a concentration process (Fig. 1). Under these conditions, despite a low amount of bacteria might be killed throughout the mechanical and chemical effects of ultrasounds, the majority of microorganisms remain viable and are able to grow in solid media (Monsen et al. 2009).

In addition, it has been reported that the shape of bacteria might have a significant effect on their sensitivity to ultrasonic treatments. Generally, large bacteria are more sensitive to sonication than small bacteria because of the large surface area exposed to ultrasound. Thus, cocci/spherical bacteria are more resistant to sonication than bacilli/rod shaped bacteria (Joyce 2003). In particular, Gram-negative bacteria seem to be more susceptible to the detrimental effects generated by ultrasounds (cell wall thinning of cell membranes, localised heating and production of free radicals) than Gram-positives due to the lack of a thick and robust cell wall (Piyasena et al. 2003).

3 Breast Implants

Breast implants are increasingly used for aesthetic reasons or in patients after mastectomy (Cook and Perkins 1996; Herdman and Fahey 2001). Although infection occurs in 1.1–2.5 % after aesthetic breast augmentation and up to 35 % after breast implant reconstruction following mastectomy (Washer and Gutowski 2012), common complications after breast surgery with prosthesis implantation are capsular fibrosis and capsular contracture (Spear and Baker 1995).

The aetiology of capsular contracture remains still unclear. Different hypotheses are considered; however, many authors postulated that bacterial colonization and biofilm formation by coagulase-negative Staphylococci (CoNS), *Propionibacterium acnes* and other skin-flora microorganisms might lead to chronic inflammation and subsequent implant fibrosis (Del Pozo et al. 2009; Portillo et al. 2013; Rieger et al. 2014).

Although several authors investigated the role of sonication in determining whether capsular contracture was associated with bacterial colonization, only few studies included subjects with breast implant infection (Table 1).

In 2013, Rieger et al. performed a multicentric study with the aim of investigating the association between the presence of capsular contracture

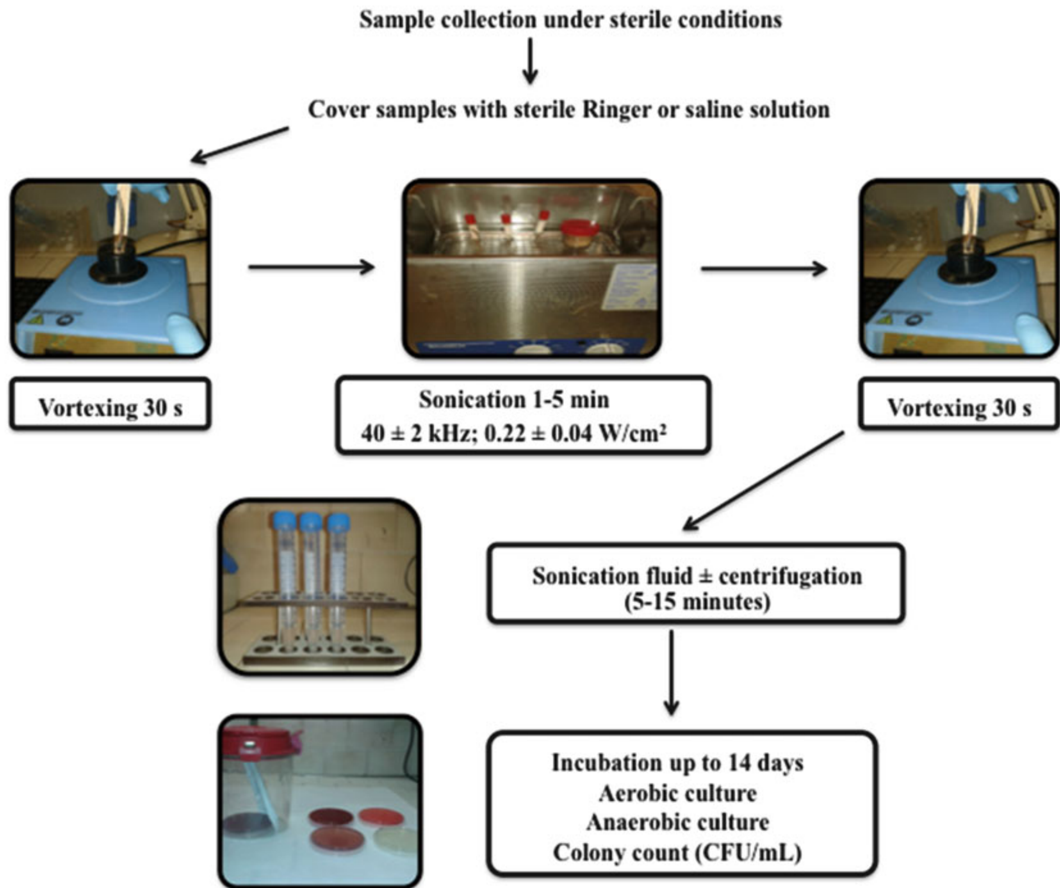

Fig. 1 Sonication protocols for the microbiological diagnosis of Implant-Associated Infections (IAIs), with or without the centrifugation as a concentration process (Tande 2014; Oliva 2013)

and bacterial biofilms on the surface of 121 removed implants. A strong correlation between the degree of capsular contracture and positive sonication culture was shown. Interestingly, all the 9 subjects who had clinical signs of breast infection yielded bacteria with a bacterial amount $>10^3$ CFU/mL, suggesting that a colony count cut-off value might be used to distinguish between colonization and infection in the setting of breast implants (Rieger et al. 2013).

A subsequent study (Karau et al. 2013) prospectively included 328 breast tissue expanders removed for any reason including infection; although the infected subjects were few ($n = 7$), in this subgroup the sonication showed higher sensitivity than tissue cultures.

Therefore, these studies showed that breast prostheses could be asymptomatically colonised by microorganisms producing biofilm, thus leading to chronic inflammation and capsular contracture. In fact, biofilm-embedded microorganisms are able to evade phagocytosis and cause persistent low-grade infection because IgG and complement deposition is diminished on the surface of foreign devices covered by bacteria (Zimmerli and Sendi 2011).

Whether or not the presence of bacteria on breast implant surface of clinically uninfected subjects is a risk factor for future infection or capsular contracture remains unknown. Additional studies investigating the role of bacterial colonization in determining or facilitating

Table 1 Studies investigating the role of sonication of breast implants

Author, year	Type of devices, n	Clinic	Baker class, n (%)	Duration of sonication procedure	Frequency/ power	Vortexing/ shaking	Rate of bacterial detection (SC), n (%)	Comparison with TC	Rate of bacterial detection (TC), n (%)	Microbiology of SC[a]	Quantification of bacteria
Pajkos et al. (2003)	Capsules, 27	No infection	19 (70): III–IV	20 min	42–47 kHz/ not specified	Yes (3 min)	17/19 (89.5): III–IV	Yes[b]	0/27 (0)	CoNS (15)	Yes
										Bacillus spp. (2)	
			8 (30): I–II				1/8 (12.5): I–II			*P. acnes* (2)	
Pajkos et al. (2003)	Implants, 21	No infection	13 (62): III–IV	20 min	42–47 kHz/ not specified	Yes (3 min)	5/13 (38.5): III–IV	Yes[b]	0/21 (0)	CoNS (3),	Yes
										Bacillus spp. (2),	
			8 (38): I–II				1/8 (12.5): I–II			*P. acnes* (1)	
Del Pozo (2009)	Implants, 45	No infection	27 (60): III–IV	5 min	40+/−2 kHz; 0.22+/ −0.04 W/cm^2	Yes (30 s)	9/27 (33): III–IV	Yes	12/27 (44): III–IV	*Propionibacterium* spp. (7),	Yes
			18 (40): I–II							CoNS (5)	
							1/18 (5): I–II		2/18 (11): I–II	*Corynebacterium* spp. (1)	
Rieger (2009)	Implants, 22	No infection	III–IV	5 min	40+/−2 kHz; 0.22+/ −0.04 W/cm^2	Yes (30 s)	9/22 (41)	No	Not applicable	CoNS (7), *Propionibacterium* spp. (6)	Yes
Rieger (2013)	Implants, 89	No infection	21 (23): I–II	1 min	40 kHz/ 0.22 W/cm^2	Yes (30 s)	4/21 (19): I–II	No	Not applicable	*P. acnes* (18), CoNS (16), *Bacillus* spp. (3)	Yes
			68 (76): III–IV				36/68 (53): III–IV			*Candida* spp. (1)	
										Others (2)[c]	
Rieger (2013)	Implants, 9	Infection	Not reported	1 min	40 kHz/ 0.22 W/cm^2	Yes (30 s)	9/9 (100)	No	Not applicable	*S. aureus* (3), CoNS (3), *P. acnes* (2)	Yes
										Citrobacter koseri (1)	
Karau (2013)	Breast tissue expanders, 321	No infection	Not reported	5 min	40+/−2 kHz; 0.22+/ −0.04 W/cm^2	Yes (30 s)	52/321 (16.2)	Yes	37/321 (11)	*Propionibacterium* spp. (45), CoNS (10)	Yes
										Others (5)[d]	
Karau (2013)	Breast tissue expanders, 7	Infection	Not reported	5 min	40+/−2 kHz; 0.22+/ −0.04 W/cm^2	Yes (30 s)	6/7 (85.7)	Yes	4/7 (57)	CoNS (5)	Yes
										P. acnes (1)	
										Serratia marcescens (1)	

SC sonication culture, *TC* traditional culture, *CoNS* coagulase-negative Staphylococci
[a]Polymicrobial growth is included
[b]TC means swab
[c]Others include: *Corynebacterium* spp. (1), *Finegoldia magna* (1)
[d]Others include: *Corynebacterium* spp. (2), *Actinomyces neuii* (1), *Pandoraea* spp. (1), *Ralstonia pickettii* (1)

subsequent capsular contracture are needed. In this setting, the use of sonication method might represent an essential tool.

4 Urinary Tract Implants

During the last decades, the incidence of catheter-associated urinary infections has increased, representing almost 40 % of nosocomial infections in catheterized patients (Holà et al. 2010). The risk of infection has been shown to be dependent on the length of catheterization (Paick et al. 2003; Tenke et al. 2006).

So far, only few authors have investigated the role of sonication method in the detection of microbial growth on the surface of ureteral stents and urinary catheters. Furthermore, most of the studies focused on bacterial colonization rather than infection of devices (Table 2).

Holà et al., who performed a study in order to investigate the biofilm microbial diversity of 535 catheters, was able to isolate a higher number of microorganisms throughout the use of sonication than throughout conventional (urine) culture. Of note, most of the catheters showed polymicrobial growth (Holà et al. 2010).

The results of this study were partially confirmed by Bonkat et al. who investigated the colonization rate of suprapubic catheters (SC). The authors found that sonicate-fluid culture was equally sensitive as urine culture in detecting bacterial colonization, with an increased rate of colonization if the device was *in situ* for more than 14 days. Similarly to other authors, polymicrobial bacterial detection was prevalent and sonication fluid showed the ability to detect more microorganisms than urine culture (Bonkat et al. 2013b). However, whether the presence of microorganisms in suprapubic catheters might represent a predisposing condition for subsequent infections should be further assessed.

In order to evaluate the potential role of sonication in the detection of microbial ureteral stent colonization (MUSC), the same group (Bonkat et al. 2011) made a prospectic study including a total of 408 ureteral stents removed for any reason. Sonication fluid culture showed higher sensitivity in detecting MUSC than traditional culture.

Subsequently, a prospectic randomised study performed in order to compare the roll-plate with the sonication technique in detecting MUSC was conducted by randomly allocating 271 ureteral stents to one of the two aforementioned methods (Bonkat et al. 2013a). In comparison with urine cultures, both roll-plate and sonication resulted in a significantly higher detection rate of colonization. Surprisingly, roll-plate showed a statistical significant higher bacterial detection than sonication whereas sonication was confirmed to be more efficient in identifying mixed biofilms. According to the results of the study, the authors postulated that sonication should not be regarded as the diagnostic procedure of choice for studying MUSC, because it required additional technical equipment, was not cost-effective and not able to identify a greater number of microorganisms than roll-plate method.

Higher sensitivity of sonication in detecting microorganisms from both inner and outer surface of implants has been previously postulated (Cozzaglio et al. 1997), due to the fact that sonication, by radiating in a liquid medium, should uniformly dislodge bacteria from the biofilm whereas roll-plate, by rolling the external surface of the implant on the agar plate, should detect only bacteria present on the external surface. However, this advantage has not been observed in the studies investigating urinary tract catheters and ureteral stents (Barford et al. 2008).

Based on the studies performed so far, sonication did not show any advantage over traditional cultures in detecting bacteria on urinary tract implants.

5 Neurosurgical Devices

EVD and VPS are increasingly used for the treatment of acute and chronic hydrocephalus. Bacterial colonization of these catheters might occur, with subsequent catheter obstruction, infection, or both (Lo et al. 2007; Beer et al. 2008; Hoefnagel et al. 2008).

Table 2 Summary of studies evaluating the role of sonication in the setting of urinary implants (urinary catheters, suprapubic catheters, ureteral stents)

Author, year	Type of devices, n	Clinic	Duration of sonication procedure	Frequency/power	Vortexing/shaking	Rate of bacterial detection (SC), n (%)	Comparison with TC	Rate of bacterial detection (TC), n (%)	Microbiology of SC	Quantification of bacteria
Holà (2010)	Urinary catheters, 535	Not reported	10 min	Not specified/not specified	Yes (2 min)	Not reported[a]	Yes	Not reported[a]	*Enterococcus faecalis* (294), *Escherichia coli* (213), *Pseudomonas aeruginosa* (149), *Candida albicans* (141) Others (758)	No
Bonkat (2013)	Suprapubic catheters, 209	Any reason for removal (n = 209)	1 min	40+/−2 kHz; 0.22+/−0.04 W/cm^2	Yes (30 s)	199/209 (95)	Yes	199/209 (95)	*Enterobacteriaceae* (196), *Enterococcus* spp. (110), *Pseudomonas* spp. (44), Others (78)	Yes
Bonkat (2013)	Suprapubic catheters, 22	Infection	1 min	40+/−2 kHz; 0.22+/−0.04 W/cm^2	Yes (30 s)	22/22 (100)	Yes	22/22 (100)	Not specified	Yes
Bonkat (2011)	Ureteral stents, 408	No infection	1 min	40+/−2 kHz 0.22+/−0.04 W/cm^2	Yes (30 s)	145/408 (36)	Yes	60/408 (15)	CoNS (41), *Enterococcus* spp. (40), *Enterobacteriaceae* (38) Others (105)	Yes
Bonkat (2013)	Ureteral stents, 271	No infection	1 min	40+/−2 kHz; 0.22+/−0.04 W/cm^2	Yes (30 s)	77/271 (28)	Yes	96/271 (35)[b]	*Enterococcus* spp. (23), *Candida* spp. (19) *Enterobacteriaceae* (16), CoNS (15) Others (36)	Yes

SC sonication culture, *TC* traditional culture (urine culture), *CoNS* coagulase-negative Staphylococci
[a]The authors identified a total of 1555 and 727 different strains throughout SC and TC, respectively
[b]TC means roll-plate method

The suspicion of catheter-associated infection is confirmed if ventricular cerebrospinal fluid (CSF) cultures are positive (Horan et al. 2008), irrespective of the presence of bacteria on the explanted ventricular catheter tips or VPS (Mayhall et al. 1984; Lozier et al. 2002).

However, recent data showed that sonication of neurosurgical devices was associated with a significantly higher rate of bacterial growth than CSF cultures (Jost et al. 2014) (Table 3), especially in subjects with EVD and VPS filling the CDC criteria for meningitis. The authors suggested that sonication of neurosurgical devices might represent a potential and useful aid for the diagnosis of meningoventriculitis. Most important, the development of clinical significant meningitis might be anticipated by the positivity of EVD or VPS sonication culture, thus highlighting the potential role of this method in the diagnostic algorithm of infections associated with EVD/VPS.

A previous study investigating the rate of bacterial colonization in cerebral catheters by using roll-out or sonication method found that both antibiotic-impregnated and non-impregnated catheters were colonized whereas CSF cultures were positive only in a minority of patients (Zabramski et al. 2003). However, the authors neither specified the precise protocol used neither the number of catheters tested with roll-plate or sonication.

Other authors investigated the rate of bacterial colonization on catheter tips by adapting for cerebral catheters the sonication technique described for vascular catheter cultures (Sherertz et al. 1990). They found colonization of silver-impregnated catheters whereas all the corresponding CSF cultures were negative (Lackner et al. 2008).

6 Endovascular Implants

6.1 Vascular Grafts

Due to the high occurrence of cardiovascular diseases, there has been a growing use of vascular (peripheral and/or aortic) grafts (Darouiche 2004). Although representing a rare event, vascular graft infections are associated with high morbidity and mortality (Calligaro et al. 2003; Saleem et al. 2010).

Because bacteria isolated from superficial or deep wounds might represent skin flora colonization, obtaining cultures from the explanted graft appears essential. However, broth cultures might be hampered by a previous antimicrobial therapy (FitzGerald et al. 2005; Stone et al. 2008); in this setting, the application of sonication method had been described (Table 4).

In fact, the importance of combining a method able to disrupt biofilm in combination with traditional broth culture in the setting of vascular graft infections has been shown in a canine model of *S. epidermidis* infection since almost 30 years (Bergamini et al. 1989).

Subsequent studies investigating the sonication of vascular graft implants were mainly based on animal models. Only one study dated 1987 evaluated the recovery of bacteria in both a canine model and in 7 graft materials excised from patients undergoing femoral anastomotic pseudoaneurysm repair. The authors found that sonication significantly increased the incidence of positive cultures from excised graft material compared with conventional culture methods (Tollefson et al. 1987).

6.2 Cardiac Devices

The growing use of implantable cardiovascular devices [permanent pacemakers (PPM), implantable cardioverter-defibrillators (ICD)] for the treatment of arrhythmias and heart failure has led to a rising incidence of CDIs over the time (Athan 2014), with an estimated rate of infections ranging from 0.13 to 19.9 % (Voigt et al. 2010; Lekkerkerker et al. 2009). Traditional cultures showed low sensitivity and specificity for diagnosing CDIs (Chua et al. 2005), mostly due to biofilm formation on cardiac devices surface (Lekkerkerker et al. 2011). In contrast, the sonication method demonstrated a higher sensitivity than conventional cultures in the microbiological diagnosis of CDIs (Oliva et al. 2010; Rohacek et al. 2010; Oliva et al. 2013) (Table 4).

Table 3 Summary of studies analyzing the sonication of cerebral catheters

Author, year	Type of cerebral devices	Clinic	Duration of sonication procedure	Frequency/ power	Vortexing/ shaking	Bacterial growth (SC) n, (%)	Microbiology of SC	Quantification of bacteria	Bacterial growth (CSF) n, (%)	Microbiology of CSF culture
Jost (2014)	EVD (n = 5)	Meningitis	1 min	40 ± 2 kHz/ 0.22 ± 0.04 W/ cm²	Yes (30 s)	4 (80)	CoNS (3), CoNS + *Corynebacterium* spp. (1)	Yes	2 (40)	CoNS (2)
Jost (2014)	EVD (n = 9)	No infection	1 min	40 ± 2 kHz/ 0.22 ± 0.04 W/ cm²	Yes (30 s)	5 (55)[a]	CoNS (5)	Yes	2 (22)[b]	CoNS (1), *P. acnes* (1)
Jost (2014)	VPS (n = 6)	Meningitis	1 min	40 ± 2 kHz/ 0.22 ± 0.04 W/ cm²	Yes (30 s)	6 (100)	CoNS (3), *E. coli* (1), *Enterobacter cloacae* (1), *Ps. aeruginosa* (1)	Yes	5 (83)	CoNS (2), *E. cloacae* (1), *E. coli* (1), *Ps. aeruginosa* (1)
Jost (2014)	VPS (n = 7)[c]	No infection	1 min	40 ± 2 kHz/ 0.22 ± 0.04 W/ cm²	Yes (30 s)	5 (83)	CoNS (3), *S. aureus* (1), *P. acnes* (1)	Yes	0 (0)	0
Zabramski (2003)	Antibiotic-impregnated EVD catheters (n = 123)	No infection	Not specified	Not specified	Not specified	22 (17.9)	CoNS (10), uncharacterized Gram positive cocci (3), *Pseudomonas* spp. (3), *Corynebacterium* spp. (2), others (7)[d]	Yes	2/149 (1.3)	*E. faecalis* + *S. aureus* (1), *E. aerogenes* (1)
Zabramski (2003)	Non antibiotic-impregnated EVD catheters (n = 109)	No infection	Not specified	Not specified	Not specified	40 (36.7)	CoNS (33), uncharacterized Gram positive cocci (6), *Corynebacterium* spp. (3), *S. aureus* (2), others (9)[e]	Yes	13/139 (9.4)	CoNS (8), *Acinetobacter calcoaceticus* + *Klebsiella pneumoniae* (1), CoNS+ *Corynebacterium* sp. (1), *Corynebacterium* sp. (1), CoNS+ *E. aerogenes* (1), *S. aureus* (1)
Lackner (2008)	Silver-impregnated EVD catheters (n = 19)	No infection	1 min	55 kHz/125 W	Yes (15 s)	5 (26)	CoNS (5)	No	0 (0)	0

EVD external ventricular drains, *VPS* ventriculo-peritoneal shunts, *CSF* cerebrospinal fluid, *CoNS* coagulase-negative Staphylococci

[a]2 out of 9 patients with EVD without meningitis but with positive sonication fluid culture eventually needed antimicrobial therapy because of a subsequent development of meningitis

[b]Microorganisms in CSF culture were detected only after culture enrichment and considered as contamination

[c]Devices (7) were collected from 6 patients

[d]Others include *E. coli* (1), *E. aerogenes* (1), uncharacterized Gram-positive rods (1), Group D *Enterococcus* spp. (1), *S. capitis* (1), *S. caprae* (1), yeast (1)

[e]Others include *Bacillus* spp. (2), *Pseudomonas* spp. (2), *Acinetobacter calcoaceticus* (1), *A. baumanii* (1), *E. agglomerans* (1), *Micrococcus* spp. (1), uncharacterized Gram-negative rods (1)

Table 4 Studies investigating the role of sonication of endovascular implants (vascular grafts, cardiac devices, CVC)

Author, year	Type of implant	Clinic	Duration of sonication procedure	Frequency/ power	Vortexing/ shaking	Rate of bacterial detection (SC), n (%)	Comparison with standard culture	Rate of bacterial detection (TC)[a], n (%)	Quantification of bacteria
Bergamini (1989)	dacron grafts	Canine model	Not applicable	Not applicable	Not applicable	30/36 (83)	Yes	26/36 (72.2)	No
Tollefson (1987)	Vascular grafts	Canine model + infected humans (n = 7)	Not applicable	Not applicable	Not applicable	7/7 (100)	Yes	Not applicable	No
Wengrovitz et al. (1991)	PTFE + knitted Dacron grafts	*In-vitro* model	Not applicable	Not applicable	No	Not applicable	Yes	Not applicable	Yes
Schmitt et al. (1986)	ePTFE, woven Dacron + velour knitted Dacron	*In-vitro* model	Not applicable	Not applicable	No	Not applicable	No	Not applicable	Yes
Oliva (2010)	Cardiac devices[b]	Infection (n = 1)	5 min	>20 kHz/not specified	Yes (30 s)	1/1 (100)	Yes	0/1 (0)	No
Oliva (2013)	Cardiac devices[b]	Any reason (n = 40); Infection (n = 20)	5 min	>20 kHz/not specified	Yes (30 s)	18/20 (90)	Yes	16/20 (80)	Yes
Rohacek (2010)	Cardiac devices	Any reason (n = 121); Infection (n = 6)	1 min	40 ± 2 kHz/ 0.22 ± 0.04 W/ cm^2	Yes (30 s)	50/121 (41.3)	Yes	34/118 (28.8)	Yes
Mason (2011)	Cardiac devices	Any reason (n = 82); Infection (n = 16)	5 min	42 ± 6 % kHz/ not specified	No	26/82 (31.7)	Yes	21/82 (25.6)	No

Viola et al. (2009)	Cardiac devices[b]	Battery failure (n = 20)	5 min	Not specified/ not specified	Yes (30 s)	Not applicable	Yes	Not applicable	Yes
Sherertz (1990)	CVC	Consecutive removed CVC (n = 1681)	1 min	55 kHz/125 W	Yes (15 s)	774/1681 (46)	No	Not applicable	Yes
Sherertz (1997)	CVC	Consecutive removed CVC (n = 89)	1 min	55 kHz/125 W	Yes (15 s)	26/45 (57.8)[c]	Yes	17/45 (37.8)	Yes
Bouza (2005)	CVC	Consecutive removed CVC (n = 1000); Infection (n = 82)	1 min	55 kHz/125 W	Yes (15 s)	313/1000 (31.3)	Yes	326/1000 (32.6)	Yes
Slobbe (2009)	CVC	Consecutive removed CVC (n = 313); Infection (n = 89)	1 min	23 kHz/Not specified	Yes (15 s)	53/313 (16.9)	Yes	66/313 (21)	Yes
Guembe (2012)	Long-term CVC	Consecutive removed CVC (n = 149); Infection (n = 11)	1 min	55 kHz/125 W	Yes (15 s)	17/149 (11.4)	Yes	37/149 (24)	Yes

SC sonication culture, *TC* traditional culture, *PTFE* polytetrafluoroethylene, *ePTFE* expanded polytetrafluoroethylene, *CVC* central venous catheters
[a]TC means roll-plate method
[b]Cardiac device means both generators and atrial/ventricular leads
[c]The sensitivity of sonication culture was calculated on 45 catheters

A recent study conducted by our group showed that, among 20 subjects with clinically defined infection, sonication culture was positive in 18/20 patients (90 %) whereas traditional culture and intraoperative pocket swab only in 16/20 (80 %) and 6/20 (33 %), respectively. When the components of cardiac devices were analyzed (generators plus electrodes), culture after sonication yielded bacteria in 77 % of the components (46/60) compared with 60 % (36/60) by standard culture. Not surprisingly, the most isolated microorganisms were CoNS and polymicrobial infections were found in 25 % of the subjects. In order to investigate the role of sonication in the setting of asymptomatic bacterial colonization, we included in the study 20 additional subjects without infection: sonication fluid culture was positive in 8 patients (40 %) whereas traditional culture of device was positive in only 4 cases (20 %). We concluded that sonication showed higher sensitivity in pathogen detection compared with traditional culture, both in infected and non-infected cardiac devices (Oliva et al. 2013). In addition, we speculated that the differences in pathogen recovery between generators and electrodes could have been explained by the different characteristics of generators and electrodes in terms of material, surface and position (Merritt et al. 1998; Clauss et al. 2010).

Rohacek et al., who compared traditional swab cultures with sonication in 121 intracardiac devices, found that, among 6 subjects with clinically defined infection, sonication fluid grew bacteria in 6/6 compared to 4/6 in swab cultures; in contrast, among 115 subjects without infections, 44/115 (38 %) sonicate fluids and 30/112 (27 %) swab cultures were positive for bacterial growth.

Mason et al. (2011) demonstrated that ultrasonication of PPM and ICD generators increased the diagnosis of pocket infection over tissue culture and swab culture alone. By using a 5 min sonication-protocol without vortexing, the authors found that, out of 82 patients with PPM or ICD undergoing generator explantation for elective reasons (n = 66) or for pocket infection (n = 16), sonication fluid yielded bacteria in 26/82 (31.7 %) whereas tissue and swab cultures were positive in 21/82 (25.6 %) and 13/82 (15.8 %), respectively.

The latter two studies (Rohacek et al. 2010; Mason et al. 2011) found *P. acnes* as a leading pathogen implicated in asymptomatic bacterial colonization and, to a lesser extent, in infection. *P. acnes*, which is part of the normal human microbiota, has been recognized as a cause of different types of IAIs, including breast prosthesis (Del Pozo et al. 2009; Rieger et al. 2009), neurosurgical shunts (Conen et al. 2008), cardiovascular devices (Delahaye et al. 2005; Lalani et al. 2007), ocular (Deramo and Ting 2001) and orthopedic implants (Piper et al. 2009; Haidar et al. 2010). The discrepancies in the rate of *P. acnes* identification between different studies might rely on the difficulties in culturing this pathogen, which has been shown to require a 14-days aerobic and anaerobic incubation in order to optimize its detection.

On the other hand, Viola et al. reported that culture alone with incubation of cardiac devices for 24 h showed results comparable with those obtained through a combination of different diagnostic methods such as sonication and vortexing (Viola et al. 2008). They performed exclusively an *in-vitro* study with 20 sterilized PPM and leads that had been removed from patients because of battery failure and incubated with a biofilm-producing clinical strain of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*. Different culturing methods such as incubation alone, vortexing followed by incubation, sonication followed by incubation, vortexing plus sonication followed by incubation were compared. The conclusion of the authors was that incubation alone was more than adequate for culturing cardiac devices; however, the results of this study might have been affected by the fact that it was performed only *in-vitro* whereas the aforementioned experiences proved the efficacy of sonication by applying it in patients with and without infection.

Although in the literature no data could be found regarding the sonication of cardiac devices other than PPM and ICD, it could be supposed that biofilm formation on the surface of heart

valves might interfere with the microbiological diagnosis of prosthetic valve endocarditis, especially in patients receiving antimicrobial therapy. Thus, the potential usefulness of the sonication method in this setting might be considered and deserves further studies.

6.3 Central Venous Catheters

Catheter-related bloodstream infections (C-RBSI) are common nosocomial infections occurring mostly in critically-ill patients, with an incidence of 2.79 per 1.000 catheter-days (Lorente et al. 2005).

Sonication has been widely applied on both long and short-term CVC and is mentioned by Infectious Diseases Society of America (IDSA) current guidelines of Intravascular Catheter-Related Infection as a feasible diagnostic procedure together with the roll-plate technique described by Maki (Mermel et al. 2009). The guidelines recommendation on the use of sonication method in the setting of C-RBSI is mainly based on the results of studies performed in the 90s (Sherertz et al. 1990) (Table 4).

However, there is a clear agreement that roll-plate culture is as accurate as sonication for the diagnosis of catheter-related infections because it is easier, faster and shows a better cost-efficiency profile and less risk of contamination than sonication (Bouza et al. 2005; Slobbe et al. 2009; Erb et al. 2014). Thus, the Maki method is currently used in the routine microbiological diagnosis of C-RBSI.

The rationale of preferring sonication is mainly based on the fact that CVC infections are caused by well-known biofilm producing microorganisms such as CoNS, *S. aureus* (McCarthy et al. 2015) and *Candida* spp. (Tumbarello et al. 2012b). In addition, roll-plate method might occasionally give false-negative results for patients receiving antimicrobials and whose mechanism of colonization is supposed to be endoluminal.

Supporting this concept, in 1997 a clinical trial reported that, compared to roll-plate and flushing methods, sonication of the subcutaneous segment and tip was the most sensitive technique for detecting catheter colonization (Sherertz et al. 1997).

Subsequently, large prospectic and randomized studies have investigated whether sonication was more sensitive than roll-plate in the diagnosis of catheter infection or colonization (Bouza et al. 2005; Slobbe et al. 2009; Guembe et al. 2012; Erb et al. 2014).

Bouza et al. compared vortexing, sonication and roll-plate in 1000 catheter tips. Although the differences were not significant, Maki's technique had higher sensitivity than sonication and vortexing, especially for short-term catheters. Slobbe et al., who randomized 313 catheter tips to be sonicated and cultured with roll-plate technique, found that roll-plate tip culture was positive in 66/313 (21.1 %) whereas only 53/313 (16.9 %) yielded bacteria with sonication. In particular, 89/313 (28.4 %) catheters were removed because of clinical suspicion of C-RBSI and/or exit site infection with concomitant bacteremia; in this subgroup, both methods showed low sensitivity and high specificity.

However, it has to be pointed out that in this study all catheter tips underwent both methods but were randomized to one method first. Some authors postulated that when one method is performed first, the subsequent use of the same sample might affect the sensitivity of the second method (Sherertz et al. 1997; Erb et al. 2014). This assumption was confirmed by this study, where both sonication and roll plate resulted less sensitive when performed in second instance.

In contrast to roll plate method, which is considered able to dislodge bacteria only from the extra-luminal surface, the sonication technique is able to disrupt the whole biofilm on foreign body and detect bacteria from both the endoluminal and exoluminal surfaces. Thus, even if sonication might be considered the best diagnostic method due to the hypothesis that the route of CVC infections is thought to be more often endoluminal, this technique did not show any advantage over roll-plate method. Rather, it appeared to be less cost-effective and more prone to contamination during sample processing than Maki method.

7 Other Implants

Theoretically, each type of implant could lead to biofilm formation. Although some *in-vitro* studies have used the sonication in order to evaluate the bacterial adherence to intraocular lens (Schauersberger et al. 2003), to our knowledge no studies investigating the role of this technique in the diagnosis of colonization or infection of penile, tracheal, intraocular and acoustic prostheses have been performed so far. However, the potential usefulness of the sonication method in these settings might be taken into consideration and deserves further investigations.

8 Clinical Implications of Sonication Method

In the present review, the use of sonication of several implants other than orthopedics has been described. However, an additional value of sonication method could be recognized especially in the diagnosis and pathogenesis of cerebral and cardiac devices infections.

In fact, the study conducted by Jost and colleagues provided information about the usefulness of bacterial quantification in the sonication fluid, suggesting that an EVD/VPS culture with more than 50 CFU might raise the suspicion of meningitis, even if the CSF cultures are negative. Additionally, they speculated that a lower number of bacteria found in the sonication fluid might represent an early condition in the development of infection, thus providing new insights on the pathogenesis of EVD/VPS infections (Jost et al. 2014).

A previous study conducted by our group on subjects with clinically defined CDIs showed that bacterial growth was observed in 65 % of the leads, even in the absence of visible vegetations seen at echocardiography, which is considered to be the most reliable method to identify endocarditis on electrodes, tricuspid valve, or both. These findings, together with the fact that the majority of cultured microorganisms were part of skin flora, were consistent with the pathogenetic hypothesis of wound contamination at the time of implantation or during the device procedure, which might facilitate bacterial colonization of generator pocket and subsequent migration along the intravascular components of the system (Oliva, submitted). Thus, the concept that intracardiac electrodes are colonized by bacteria without visible vegetation might lead to new insights on the early recognition of subjects at major risk of developing endocarditis compared to those who only develop pocket infection.

Furthermore, it has been shown that the sensitivity of sonication fluid is less hampered by antimicrobial therapy than conventional cultures. In contrast to PJIs, where antimicrobial therapy might be stopped at least 2 weeks before prosthesis explanation in order to obtain the highest bacterial yield (Trampuz et al. 2007), subjects with cerebral or cardiac implants are more likely to be on antimicrobial therapy when the device is removed. Thus, the use of a diagnostic method which is minimally affected by antimicrobial therapy appears to be critical.

In fact, the potential effect of antimicrobial therapy on the diagnostic sensitivity of CSF culture might lead to additional difficulties in the interpretation of clinical and laboratory parameters for the diagnosis of meningoventriculitis. Despite the study population was small, the encouraging results of the study conducted by Jost and colleagues might be useful in the early identification of patients with EVD or VPS at high risk of developing meningitis.

In the setting of CDIs, the usefulness of sonication in subjects receiving antimicrobial therapy at the time of device removal has been investigated in a previous study performed by our group. Despite in subjects on therapy >14 days bacterial growth was lower than in subjects who were on therapy <14 days, the difference was not statistically significant, thus highlighting that sonication might retain its diagnostic value in the presence of antimicrobials (Oliva et al. 2013).

9 Conclusions

IAIs are difficult-to-treat infections associated with high morbidity, mortality and length of hospitalization. They are characterized by biofilm

formation on implant surface, which leads to the difficulty in microbiological diagnosis and the need of device removal. The application of sonication method might represent an essential tool in order to improve the microbiological diagnosis in the setting of IAIs other than PJIs whereas the assumption that sonication might have additional diagnostic advantage over traditional culture in urinary tract implants has not been confirmed so far. The potential usefulness of the sonication in the setting of other implants such as heart, penile, tracheal, intraocular and acoustic prostheses might be taken into consideration and deserves further investigations. Moreover, the possibility to perform additional studies including molecular and/or immunological analyses on the sonication fluid might give physicians valuable insights into both IAIs pathogenesis and detection of fastidious microorganisms such as *P. acnes*.

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The Isolation, Identification and Analyses of *Lactobacillus* Genus Bacteria with Probiotic Potential

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Abstract

103 strains of lactic acid bacteria of *Lactobacillus* genus were isolated from natural sources and identified for genus and species level with API tests and 16S rRNA sequencing. However, only 27 strains from isolated cultures demonstrated a high stability to gastric stress and from that – only 15 strains were highly resistant to intestinal stress. Results indicated that only some isolated cultures of lactobacilli possessed potential probiotical properties and could serve as new probiotics for dairy industry with high resistance to gastro-intestinal stresses.

Keywords

Isolation of lactic acid bacteria • Molecular identification • Probiotic properties • Gastro-intestinal stress

1 Introduction

Scientific interest to lactic acid bacteria (LAB) emerged at the end of nineteenth century. Nowadays, live microorganisms if used in right quantities and benefit to host organism's health were defined as probiotics (Reid et al. 2003). In food industry the fortification of sour milk products with LAB probiotics was widely used (Buss 2004) that explained the interest for the studies of these microorganism survival and activity in the human intestinal tract. Food industry and Academy continued the research aimed at more profound studies of probiotic physiology and mechanisms which allowed them to benefit to human's health and wellbeing from intestinal tract (Vaughan et al. 2002; Reid et al. 2003; Boclé and Thomann 2005).

A wealth of information had been gathered over the past years on probiotics through experimental, animal and human studies, with the aim to understand the mechanism of actions and elucidate their beneficial health effects on human host. Significant amount of evidence existed for their ability to increase the bioavailability of minerals and to stimulate the immune system, although there were less clear evidences so far for their

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prophylactic or therapeutic role in gastrointestinal infections. Moreover, the effect of the food delivery vehicle on the efficacy of probiotics was an area that had been hardly investigated (Charalampopoulos and Rastall 2011). It was demonstrated that food-grade LAB could be used for the interacting with the human gut with benefits to its health (Sanchez et al. 2011).

Plenty of LAB strains were isolated from different sources: human milk (Langa et al. 2012) and dairy products (Bujňáková and Kmeť 2012), Italian and Argentinean cheeses (Zago et al. 2011), human and animal feces (Lee et al. 2011a; Martin et al. 2012; Zeng et al. 2011), fresh and fermented fruit and vegetable products (Kumar et al. 2012; Lee et al. 2011b; Vitali et al. 2012). Many of them had probiotic properties. They had to survive in the gastric juice and bile acids salts in the gastrointestinal tract of humans and animals (Lee et al. 2011a; Zeng et al. 2011; Jensen et al. 2012). In addition, some strains were found to be resistant to lysozyme (Kimoto-Nira et al. 2010). However, these properties were expressed in different degrees (Koll et al. 2010; Zago et al. 2011).

The aim of this study was to isolate the new LAB of *Lactobacillus* genus from different kinds of natural sources, to analyze the probiotic potential of the obtained cultures with testing of the strain resistance level to gastric and intestinal stresses.

2 Materials and Methods

2.1 Sampling

According to physiological features of LAB, four main sources for biological sampling were determined: (1) feces of adult humans and children of different ages; (2) feces of various animals; (3) hand-made dairy products from Moscow region and other areas of Russia; (4) anaerobic sludge of natural ponds and food manufacture waste waters.

2.2 Isolation of Lactobacilli from Natural Sources

MRS medium was used for the isolation of bacteria of genus *Lactobacillus* (Man et al. 1960). Isolation of pure cultures was performed in several stages.

The 1st stage – enrichment cultures. For obtaining of lactobacilli enrichment cultures, 1 g of sample was placed into 10 ml sterile serum vial, 5 ml of sterile liquid anaerobic MRS medium was added, serum vial was closed with sterile rubber stopper and fixed with aluminum crimple cap. Then, inoculated serum vial was placed into the thermostat at the temperature of 37 °C and was incubated for 1–3 days until the signs of growth appeared (dimness, change of medium color, appearance of the smell, gas formation).

The 2nd stage – isolation of pure cultures. To obtain pure culture, 0.1 ml of enrichment culture was plated onto solid MRS medium, according to Koch method, following the spreading with sterile spatula on three Petri dishes. The dishes were cultivated at the temperature of 37 °C under anaerobic conditions in anaerostat. The colonies were described, studied under microscope, their Gram stain, catalase and oxidase activities were determined. Only Gram-positive, oxidase- and catalase-negative colonies were selected; these cultures were inoculated into serum vials with liquid MRS medium. The isolate purity was confirmed by further spreading of cells on solid MRS medium to obtain single uniform colonies. In order of a long-time storage, the cultures were frozen at −70 °C in MRS broth containing 25 % (v/v) of glycerol and lyophilized in Free Zone freeze-dryer (Labconco, USA) at T = −51 °C and P = 49 kPa for 24 h. Dried cultures were kept at +4–6 °C in refrigerator.

2.3 Species Determination of *Lactobacillus*

Pure cultures of isolated LAB were tested for the usage of different carbon sources with API50CH test (BioMerieu Co., France).

Isolated cultures were further identified by partial sequencing of 16S rRNA gene and phylogenetic analysis of nucleotide sequences.

Isolation of DNA from a biomass of bacteria was done according to a technique described by Bulygina et al. (2002). Concentration of the

isolated DNA for the use of this method of was 30–50 mkg/ml.

For polymerase chain reaction and further sequencing of PCR-fragments of 16S rRNA gene, the universal primer system had been used (Lane 1991). The volume of amplification mixture was 50 μl, and it had the following composition: 1× buffer for DNA polymerase BioTaq [17 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$]; 12.5 pmol of each of dNTPs, 50 ng of DNA-matrix; 5 pmol of correspondent primers and 3 units of DNA-polymerase BioTaq (Dialat LTD, Russia). A pair of the following primers was used for amplification: 8-27f – 5-′-AGAGTTTGATCMTGGCTCAG-3′ and 1492r – 5′-TACGGYTACCTTGTTACGACTT-3′.

The temperature-time profile of PCR was as follows: the first cycle – 94 °C × 9 min, 55 °C × 1 min, 72 °C × 2 min; following by 30 cycles – 94 °C × 1 min, 55 °C × 1 min, 72 °C × 2 min; the final cycle – 72 °C × 7 min. The analysis of PCR products was performed with electrophoresis in 2 % agarose gel with electric field strength of 6 V/cm. The isolation and purification of PCR products was executed from low-melt point agarose using reagent kit by Wizard PCR Preps (Promega, USA), according to manufacturer's instructions.

The sequencing of the received PCR gene's fragments encoding 16S rRNA was performed according to Sanger's method (Sanger et al. 1977) using reagents kit of Big Dye Terminator v.3.1 (Applied Biosystems Inc., USA) on automatic sequenator ABI PRIZM 3730 (Applied Biosystems Inc., USA) according to manufacturer's instructions. The following primers were used for the sequencing: 27f 5-′-AGAGTTTGATCMTGGCTCAG, 519r 5-′-GWATTACCGCGGCKGCTG, 530f 5′-GTGCCAGCMGCCGCGG, 1114f 5-′-GCAACGAGCGCAACCC, 1492r 5-′-TACGGYTACCTTGTTACGACTT, 357f 5′-CTCCTACGGGAGGCAGCAG. The reading of amplicons was performed in two directions.

The primary analysis of 16S rRNA gene sequence of the studied strains was performed using BLAST software package (Camacho et al. 2009).

2.4 Resistance Degree Determination of Lactic Acid Bacteria of *Lactobacillus* Genus to Gastric and Intestinal Stresses

Gastric and intestinal stresses had been determined according by Pinto et al. (2006) with some modifications. The studied bacterial strains were cultivated anaerobically in MRS liquid medium, pH 6.5 for 24–48 h at 37 °C and 10 % (v/v) of CO_2.

To imitate gastric stress *in vitro*, 100 μl of the studied culture from a stationary growth phase were added to 1 ml of artificial gastric juice (dilution 1/11). The suspensions were incubated for 10, 30 and 60 min at 37 °C and 10 % CO_2. In the control sample, 1 ml of MRS was added to bacterial suspension instead of gastric juice. The tests were performed three times and the average value was calculated. After bacteria were incubated, the suspensions were diluted up to 10^{-10} in fresh MRS. The dilutions were plated to Petri dishes with MRS agar and incubated for 24–48 h at 37 °C and 10 % CO_2. The quantity of survived cells (with and without stress) was calculated according the colony counts in each dilution.

Artificial gastric juice composition contained: NaCl (Sigma S9625) – 2.2 g/l; L-lactic acid (Sigma L1750) – 9.9 g/l (0.11 M); porcine pepsin (Sigma P7125) – 3.5 g/l (600–1800 units/mg); pH 2.7 ± 0.02 (made up with 35 % NaOH); pH after dilution 1/11 was 3.10 ± 0.10 (pH was controlled for each culture).

To imitate intestinal stress *in vitro*, 100 μl of the studied culture in a steady state were added to 1 ml of artificial intestinal juice (dilution 1/11). The suspensions were incubated for 5 h at 37 °C and 10 % CO_2. In the control sample, 1 ml of MRS was added to bacterial suspension instead of intestinal juice. The tests were performed three times and the average value was calculated. After bacteria were incubated, the suspensions were diluted up to 10^{-10} in fresh MRS. The dilutions were plated to Petri dishes with MRS agar and incubated for 24–48 h at 37 °C and 10 %

CO_2. The quantity of survived cells (with and without stress) was calculated according the colony counts in each dilution.

The composition of artificial intestinal juice was as follows: bile salts (porcine bile, Sigma B8631) – 3.3 g/l (final concentration – 0.3 %), carbonate buffer $NaHCO_3$ (Sigma S8875) – 16.5 g/l (final concentration – 1.5 %); pH 6.3 ± 0.10 (pH was controlled for each culture).

The resistance degree to gastric and intestinal stresses – RD (Resistance Degree) was calculated according to formula: $RD = n_1/n_2$, where n_1 – amount of CFU in 1 ml of the control sample; n_2 – amount of CFU in 1 ml of the tested sample.

Cell resistance to gastrointestinal stresses was evaluated in the following ranges: very good – $RD \leq 5$; good – $6 < RD \leq 10$; acceptable – $11 < RD \leq 15$; unacceptable – $RD > 15$.

3 Results

3.1 Isolation and Identification of LAB

More than 1000 samples from different natural sources of biological material were analyzed. The species of 250 pure cultures of *Lactobacillus* were determined with API test. Of them, 103 strains of the isolated lactobacilli were determined as *Lactobacillus* genus bacteria and were further identified up to the species level using method of partial sequencing of 16S rRNA gene.

After identification of 16S rRNA sequences comparison, it was found those 13 strains of *L. brevis*, 9 strains of *L. paracasei*, 21 strains of *L. plantarum*, and 1 strain of each species: *L. delbrueckii*, *L. fermentum*, *L. johnsonii*, were isolated from dairy products and homemade cheeses. Two strains of *L. brevis*, 3 strains of *L. paracasei*, 10 strains of *L. rhamnosus* and 12 strains of *L. plantarum* were isolated from feces of newborns. Three strains of *L. brevis*, 3 strains of *L. paracasei*, 5 strains of *L. reuteri*, 6 strains of *L. rhamnosus*, 6 strains of *L. plantarum* and 1 strain of *L. vaginalis* were isolated from wild and homebred animal feces. Two strains of *L. paracasei* and 4 strains of *L. plantarum* were isolated from anaerobic sludge of natural ponds and food manufacture waste waters.

In total, 43 strains of *L. plantarum*, 18 – of *L. brevis*, 17 – of *L. paracasei*, 16 – of *L. rhamnosus*, 5 – of *L. reuteri* and 1 strain of each: *L. delbrueckii*, *L. fermentum*, *L. johnsonii*, *L. vaginalis* species were isolated from natural sources and homemade dairy products, which apparently reflected their spreading in natural and artificial ecological niches.

The sequences of 16S rRNA genes of 27 strains were deposited to GenBank database, where they were given numbers (Table 1).

3.2 The Determination of Resistance of Lactobacilli to Gastric and Intestinal Stresses

The tolerance of 103 isolated and determined to the species level with 16S rRNA gene sequencing *Lactobacillus* strains to gastric and intestinal stresses was studied. The results of only resistant cultures were presented in the Table 2. From 103 tested strains of lactobacilli, only 27 showed good resistance to gastric acid stress, after which they were further studied for tolerance to intestinal stress. As an example of not tolerant strains, four of them were included in Table 2 (pos. 28–31).

First of all, the isolated lactobacilli cultures were tested for the resistance to gastric juice, because bacteria in the food passed through the stomach first. *Lactobacillus* strains with good and very good resistance to gastric stress were further tested to intestinal stress influence (bile acids with pH 6.3). That corresponded to the task of this research aimed to find lactobacillus strains that could survive the passage through the stomach and duodenal intestine without considerable loss in their vitality.

During further comparison of the cultures listed in the Table 2, accordingly to the resistance to bile acids (the imitation of duodenum and upper small intestine of human gastrointestinal

Table 1 Accession numbers of the 16S rRNA sequences of isolated strains in GenBank

№	Strain name	Accession No. in GenBank
1	*Lactobacillus plantarum* CM MSU № 503	KJ160508
2	*Lactobacillus plantarum* CM MSU № 504	KJ459015
3	*Lactobacillus plantarum* CM MSU № 505	KJ459016
4	*Lactobacillus plantarum* CM MSU № 506	KJ459017
5	*Lactobacillus plantarum* CM MSU № 507	KJ459018
6	*Lactobacillus plantarum* CM MSU № 508	KJ459019
7	*Lactobacillus plantarum* CM MSU № 509	KJ459020
8	*Lactobacillus plantarum* CM MSU № 510	KJ459021
9	*Lactobacillus plantarum* CM MSU № 511	KJ459022
10	*Lactobacillus plantarum* CM MSU № 512	KJ459023
11	*Lactobacillus plantarum* CM MSU № 513	KJ459024
12	*Lactobacillus plantarum* CM MSU № 514	KJ459025
13	*Lactobacillus plantarum* CM MSU № 515	KJ459026
14	*Lactobacillus plantarum* CM MSU № 516	KJ459027
15	*Lactobacillus plantarum* CM MSU № 517	KJ459028
16	*Lactobacillus plantarum* CM MSU № 518	KJ459029
17	*Lactobacillus plantarum* CM MSU № 519	KJ459030
18	*Lactobacillus plantarum* CM MSU № 520	KJ459031
19	*Lactobacillus brevis* CM MSU № 521	KJ459032
20	*Lactobacillus brevis* CM MSU № 522	KJ459033
21	*Lactobacillus brevis* CM MSU № 523	KJ459034
22	*Lactobacillus brevis* CM MSU № 524	KJ459035
23	*Lactobacillus brevis* CM MSU № 525	KJ459036
24	*Lactobacillus brevis* CM MSU № 526	KJ459037
25	*Lactobacillus parocasei* CM MSU № 527	KJ459038
26	*Lactobacillus rhamnosus* CM MSU № 528	KJ459040
27	*Lactobacillus rhamnosus* CM MSU № 529	KJ459039

tract stress), it was noticed that among 27 cultures, showing high resistance to gastric stress, only 15 cultures had "very good" resistance level to intestinal stress, that reflected the rare occurrence of high resistance value to gastric and intestinal stresses combination.

It also should be noted that 8 of 15 strains, that demonstrated a high resistance to both kinds of stresses applied to them, belonged to *L. plantarum,* and 4 of 15 – to *L. brevis* species, this most likely reflected their confoundedness to natural niches often suffered from hostilities, such as plant surfaces, human and animal gastrointestinal tracts. It was found that *L. plantarum* cells were able to occupy all the ecological niches from where the test samples were taken: sour milk products, human and animal gastrointestinal tract (GIT) and waste water sludge, this was most likely the result of theses bacteria high resistance level to different stresses.

4 Discussion

The results of our study demonstrated that new LAB of *Lactobacillus* genus could be isolated from various kinds of natural and anthropogenic sources. This would open new horizons for the search and isolation of new LAB from many environmental niches, therefore the search for the new probiotic strains was endless. But the isolated strains were different in their survival rates toward gastric and intestinal stresses. Of 103 tested lactobacilli, only 27 strains (ca. 25 %) had shown a high resistance to stresses applied and relative good level of survival, which would make them potential probiotics. These properties would allow the further use of these cultures in functional food products, because of live cells transit through gastric and duodenum parts of GIT would not lead to considerable loss of viability of probiotic cells.

Table 2 Tolerance of the isolated *Lactobacillus* strains to gastric and intestinal stresses

№	Strains	Tolerance to gastric stress	Tolerance to intestinal stress
Strains isolated from human and animal feces			
1.	*L. plantarum* CM MSU № 503	RD = 1	RD = 9
		Very good	Good
2.	*L. plantarum* CM MSU № 504	RD = 1	RD = 4
		Very good	Very good
3.	*L. plantarum* CM MSU № 505	RD = 1	RD = 7
		Very good	Good
4.	*L. plantarum* CM MSU № 518	RD = 1	RD = 1
		Very good	Very good
5.	*L. plantarum* CM MSU № 519	RD = 1	RD = 1
		Very good	Very good
6.	*L. plantarum* CM MSU № 520	RD = 1	RD = 1
		Very good	Very good
7.	*L. rhamnosus* CM MSU № 528	RD = 1.0	RD = 1
		Very good	Very good
8.	*L. rhamnosus* CM MSU № 529	RD = 2	RD = 4
		Very good	Very good
Strains isolated from national dairy products, included in functional nutrition rations in the residence regions of persons with active longevity			
9.	*L. plantarum* CM MSU № 506	RD = 1	RD = 11
		Very good	Acceptable
10.	*L. plantarum* CM MSU № 507	RD = 2	RD = 5
		Very good	Very good
11.	*L. plantarum* CM MSU № 508	RD = 1	RD = 13
		Very good	Acceptable
12.	*L. plantarum* CM MSU № 509	RD = 1	RD = 10
		Very good	Good
13.	*L. plantarum* CM MSU № 510	RD = 1	RD = 6
		Very good	Good
14.	*L. plantarum* CM MSU № 511	RD = 1	RD = 2
		Very good	Very good
15.	*L. plantarum* CM MSU № 512	RD = 1	RD = 2
		Very good	Very good
16.	*L. plantarum* CM MSU № 513	RD = 1	RD = 12
		Very good	Acceptable
17.	*L. plantarum* CM MSU № 514	RD = 2	RD = 14
		Very good	Acceptable
18.	*L. plantarum* CM MSU № 515	RD = 1	RD = 8
		Very good	Good
19.	*L. plantarum* CM MSU № 516	RD = 1	RD = 1
		Very good	Very good
20.	*L. plantarum* CM MSU № 517	RD = 1	RD = 7
		Very good	Good
21.	*L. brevis* CM MSU № 521	RD = 2	RD = 3
		Very good	Very good
22.	*L. brevis* CM MSU № 522	RD = 1	RD = 10
		Very good	Good
23.	*L. brevis* CM MSU № 523	RD = 1	RD =2
		Very good	Very good

(continued)

Table 2 (continued)

№	Strains	Tolerance to gastric stress	Tolerance to intestinal stress
24.	*L. brevis* CM MSU № 524	RD = 1	RD = 1
		Very good	Very good
25.	*L. brevis* CM MSU № 525	RD = 1	RD = 6
		Very good	Good
26.	*L. brevis* CM MSU № 526	RD = 1	RD = 3
		Very good	Very good
27.	*L. paracasei* CM MSU № 527	RD = 1	RD = 1
		Very good	Very good
Strains isolated from anaerobic sludge			
28.	*L. paracasei* CM MSU № 531	RD = 390	RD = 80
		Unacceptable	Unacceptable
29.	*L. paracasei* CM MSU № 532	RD = 15	RD = 58
		Acceptable	Unacceptable
30.	*L. plantarum* CM MSU № 533	RD = 39	RD = 75
		Unacceptable	Unacceptable
31.	*L. plantarum* CM MSU № 534	RD = 694	RD = 38
		Unacceptable	Unacceptable

It worth to be mentioned, that among 15 strains that demonstrated high resistance to gastric and intestinal stresses, 8 strains were determined as *L. plantarum* and 4 as *L. brevis* (Table 2), that reflected their affiliation to natural environmental niches, often suffering from natural stresses – leaf surfaces, GIT of animals and humans. Strains of *L. plantarum* could inhabit all the ecological niches, we studied and sampled for the environmental probes: sour milk home-made products, animal and human GITs, waste water mud, etc. This property could put *L. plantarum* strains into the group of most resistant LAB and could be considered as valuable and most potent probiotic strains.

Taking all the aforesaid into consideration, we could prove that lactobacilli isolated in this study had a great potential for serving as probiotics, providing that they were highly resistant to gastric and intestinal stresses (Table 2). It proved that lactobacilli, isolated from natural sources, had a great potential and could be used as probiotic cultures for the treatment and preventive measures against human and animal gastrointestinal distresses.

All LAB isolated in this study from anaerobic sludge of natural ponds and food manufacture waste waters did not demonstrate resistance to gastro-intestinal stresses. As the example one can see the properties of four cultures isolated from anaerobic sludge. Four cultures isolated from sludge are not only not resistant to gastric stress, they are also very sensitive to bile acids of simulated intestine stress (Table 2). It should be also mentioned, that among these four strains two were belonging to *L. plantarum* species, which could reflect that representatives of this species could have and might not have high resistance to GIT stresses depending on the environmental probe they were isolated from and the niche they occupy in the nature.

Most strains (19 of 27) with high resistance to gastric and intestinal stresses were isolated from national diary products included into functional nutrition rations in the residence regions of persons with active longevity (Table 2). That also might reflect the applying the selective methods, which were used for thousands of years to the LAB in home-made sour milk products.

Eight strains isolated from human and animal feces show very good resistance to GIT stresses, among them 6 strains of *L. plantarum* and two – of *L. rhamnosus* species, which again put *L. plantarum* bacteria to the group of most resistant strains to gastric and intestinal environments.

It could be summarized, that potential probiotic LAB with high potential to survive GIT stresses could be isolated not only from human sources and fermented dairy products, but from natural environments with no or little anthropogenic influence. It might open a whole new large area for searching and isolation of new beneficial microbes for human and animal wellbeing and prophylaxis.

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Increase in Peripheral $CD3^{-}CD56^{bright}CD16^{-}$ Natural Killer Cells in Hashimoto's Thyroiditis Associated with HHV-6 Infection

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Abstract

Hashimoto's thyroiditis (HT) is a very common autoimmune disease of the thyroid. In addition to genetic background, several viruses, including herpesviruses, have been suggested to play a role as possible environmental triggers of disease, but conclusive data are still lacking. Previous results showed that HT patients have an increased cellular immune response directed against the HHV-6 U94 protein and increased NK activity directed against HHV-6 infected thyrocytes.

In this study, we characterized the antiviral antibody response and the NK cells activity and subtype in HHV-6 infected HT patients. The results showed that HT subjects have increased prevalence and titer of anti-U94 antibodies and a higher amount of $CD3\text{-}CD56^{bright}CD16^{-}$NK cell percentages compared to controls. Furthermore, the cell activation of $CD3^{-}CD56^{bright}$ NK cells in HT patients significantly correlates with TPO and Tg Ab levels.

The results suggest that HHV-6 might contribute to HT development, increasing NK cell secretion of inflammatory cytokines that could sustain the persistence of an inflammatory status in HT patients.

Keywords

Hashimoto's thyroiditis • HHV-6 • NK cells

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Hashimoto's thyroiditis (HT) is a very common autoimmune disease of the thyroid. In addition to genetic background, several viruses, including herpesviruses, have been suggested to play a role as possible environmental triggers of disease, but conclusive data are still lacking.

HHV-6 infection is common and has a worldwide distribution (Caselli and Di Luca 2007). Viral strains cluster in two variants that were recently classified as different viral species, on the basis of characteristic biological differences: HHV-6A, with still unknown disease association, and HHV-6B, the etiologic agent of roseola (e*xanthem subitum*), a childhood benign febrile disease. HHV-6 species *in vitro* replicate most efficiently in primary T-cells and in selected T-cell lines. However, the *in vivo* tropism of HHV-6 is considerably broader, including macrophages, endothelial cells, salivary glands, and brain (Caruso et al. 2002; Di Luca et al. 1994; Thomas et al. 2008). After primary infection, HHV-6 establishes a latent infection and resides mainly in peripheral blood mononuclear cells (PBMCs) and in macrophages (Di Luca et al. 1994; Kondo et al. 1991). During latency, HHV-6 expresses specific viral transcripts. In particular, expression of U94, in the absence of other viral lytic transcripts, is considered a molecular marker of viral latency (Caselli et al. 2006; Rotola et al. 1998).

HHV-6 has been tentatively associated to several chronic autoimmune inflammatory processes (Scotet et al. 1999), including Sjogren syndrome (Fox et al. 1989; Ranger-Rogez et al. 1994), multiple sclerosis (Alvarez-Lafuente et al. 2010; Challoner et al. 1995; Rotola et al. 2004), rheumatoid arthritis and systemic lupus erythematosus (Alvarez-Lafuente et al. 2005; Krueger et al. 1991). In addition, recent case reports suggested that HHV-6 infection might be related to the onset of autoimmune disorders, including purpura fulminans, severe autoimmune acquired protein S deficiency (Boccara et al. 2009), autoimmune connective tissue diseases (Broccolo et al. 2009), and severe autoimmune hepatitis (Potenza et al. 2008). Our analysis of fine needle thyroid aspirates (FNAs) and blood from HT patients and controls showed that HHV-6A prevalence and load are highly increased in thyroid tissue of HT patients (Caselli et al. 2012b). Furthermore, HT-derived thyrocytes harbor active HHV-6A, whereas the virus is strictly latent in the few virus-positive controls. We also reported that HHV-6A infects thyroid cells, inducing *de novo* expression of HLA-II surface antigens. Consequently, HT patients have increased CD4+ and CD8+ T-cell responses to HHV-6 U94 protein and infected thyrocytes become a target for innate Natural Killer (NK) cell killing. NK cells comprise about 10–15 % of all circulating lymphocytes and are able to lyse target cells that have lost the protective signal mediated by human leukocyte antigen (HLA) class I surface molecules (Storkus et al. 1987) as in viral infections. In particular, non-productive HHV-6A and HHV-6B infection is known to lead to the up-regulation of HLA (Human Leukocyte Antigens)-A, -B, -C molecules on dendritic cells (Bertelsen et al. 2010; Gustafsson et al. 2013), via autocrine IFN (Interferon)-α signaling, as well as the up-regulation of HLA-DR and CD86 molecules. This modification may result in the inability of NK cells to recognize target infected cells, as they still present HLA expression. Moreover, HHV-6A infection suppress DC stimulation of allogenic T cell proliferation. The ability to block innate and adaptive immune responses might be a successful strategy by which HHV-6A avoids the induction of appropriate host defense mechanisms, and thus facilitating persistent infection. Human NK cells can be divided into two subsets based on their cell-surface density of CD56 molecule in $CD56^{bright}$ and $CD56^{dim}$, each with distinct phenotypic properties. There is evidence to suggest that these NK-cell subsets have unique functional attributes and, therefore, distinct roles in the human immune response. The $CD56^{dim}$ NK cell subset is more naturally cytotoxic while $CD56^{bright}$ NK-cell subset has the ability to produce abundant cytokines following activation and has low natural cytotoxicity (Cooper and Caligiuri 2001). NK cell activities have been evaluated during HHV-6 infection. NK cell activation was high in the acute phase of HHV-6

infection and declined gradually during convalescence. These results suggest that NK cells play a major role in resolving acute phase infection while specific lymphocyte activity develops later (Kumagai et al. 2006). In this study we analyzed changes in the activity and subtype of NK cells in peripheral blood cells from HT patients.

Clinical samples derived from 8 HT patients and 8 patients with benign follicular epithelial lesions (controls). The 8 HT patients included 2 males and 6 females, with a mean age of 57 $\pm$ 15 years (range 37–78 years), with anti-thyroperoxidase antibodies (TPO Ab) $>$ 35 IU/ml (mean value = 835 IU/ml, range 343–3000 IU/ml), and anti-thyroglobulin antibodies (Tg Ab) $>$ 115 IU/ml (mean value = 205 IU/ml, range 120–366 IU/ml). The 8 control patients included 4 males and 4 females with a mean age of 64 $\pm$ 18 years (range 30–91 years) (there was no statistically significant difference between the two groups), and showed TPO Ab $<$ 35 IU/ml (mean value = 10 IU/ml, range 8–13 IU/ml), and Tg Ab $<$ 115 IU/ml (mean value = 16 IU/ml, range 11–21 IU/ml). None of the patients enrolled in the study presented other autoimmune diseases.

Patients were characterized for HHV-6 viral load in their peripheral blood mononuclear cells (PBMCs) and thyroid FNAs, obtained as part of routine clinical work from patients undergoing FNAs for diagnostic purposes, and were used after receiving approval from the Local Ethical Committee of the University of Ferrara and S. Anna Hospital of Ferrara. The patients provided written informed consent for both FNA procedure (which is part of the clinical practice) and for biomolecular analyses, to which purpose the samples were anonymized. PBMCs were isolated by Ficoll-Hypaque gradients. DNA was isolated from FNAs and PBMCs as described (Caselli et al. 2012a). HHV-6 DNA presence and load were analyzed by PCR and real time quantitative PCR (qPCR) specific for the U94 and U42 genes (Caruso et al. 2009); samples were considered positive when 1 ug of cell DNA harbored more than 100 copies of viral DNA (Caselli et al. 2012a). Amplification of the house-keeping human RNase P gene was used as a control. All clinical samples were analyzed in a randomized and blinded fashion. NK cell activity and number was measured by flow cytometer.

NK cells were characterized with a specific anti-CD panel (CD3-PerCp-Cy5.5, CD56-FITC, CD107a-PE, CD16-PE) (e-Bioscience). For the CD107a degranulation assay, that shows NK cell activation status, cells were stained with CD107a-PE (e-Bioscience) after 1 h of incubation at 37 °C and 3 h of treatment with Golgi Stop solution (Becton Dickinson) (Rizzo et al. 2012). Ten thousand events were acquired. Cell viability was assessed by propidium iodide staining. Anti-isotype controls (Exbio) were performed.

HT and control subjects were also characterized for their antibody response against HHV-6 (whole virus) or its U94/Rep protein, by testing plasma samples by specific ELISA assays (Caselli et al. 2002). As a control, the plasma samples derived from 12 healthy donors were also assayed.

The results showed that HHV-6 was more prevalent in HT FNAs (8/8, 100 %) than in FNAs derived from controls (2/8, 25 %) ($p < 0.001$) (Table 1). Furthermore, viral load was higher in HT specimens (mean 1.2×10^4 copies/µg of cellular DNA, range 8×10^2–4.7×10^4 copies/µg DNA) than in the few controls which resulted positive for HHV-6 (mean 3.9×10^2 copies/µg DNA, range 2.2–5.7×10^2 copies/µg DNA) ($p < 0.01$). Similar results were obtained in PBMCs. In particular, HHV-6 was detected in 8/8 HT PBMCs (100 %) and only in 3/8 PBMCs derived from controls (37 %) ($p < 0.01$) (Table 1). Furthermore, viral load was higher in HT specimens (mean 1.8×10^4 copies/µg of cellular DNA, range 1.8×10^2–3.9×10^4 copies/µg DNA) than in the few controls which resulted positive for HHV-6 (mean 3.7×10^2 copies/µg DNA, range 2.8–4.9×10^2 copies/µg DNA) ($p < 0.01$). Where possible, virus species characterization, performed as previously described (Caselli et al. 2012a), showed the presence of HHV-6A in the thyroid tissue and of HHV-6B in PBMCs (data not shown), confirming the

Table 1 Presence of HHV-6 in specimens obtained from HT patients and controls

Group	Sample	Virus presence[a]	Virus load (copy number/μg total DNA)[a]
HT	FNA	8/8 (100 %)	$1.2 \times 10^4 \pm 1.2 \times 10^2$
	PBMC	8/8 (100 %)	$1.8 \times 10^4 \pm 1.3 \times 10^2$
Control	FNA	2/8 (25 %)	$3.9 \times 10^2 \pm 1.3 \times 10$
	PBMC	3/8 (37 %)	$3.7 \times 10^2 \pm 1.1 \times 10$

[a]Results obtained by qPCR analysis of total DNA extracted from FNAs or PBMCs, amplifying U94 and U42 genes. Virus presence is expressed as number of positive samples on the total number of tested samples (percentage of positivity in parenthesis). Virus load is expressed as the mean value of genome copy number in the positive samples ± SE. Differences were statistically significant in HT *vs* control FNAs (100 % *vs* 25 %, $p < 0.001$) and PBMCs (100 % *vs* 37 %, $p < 0.01$). Virus load was also significantly different in HT *vs* control FNAs ($p < 0.01$) and PBMC ($p < 0.01$)

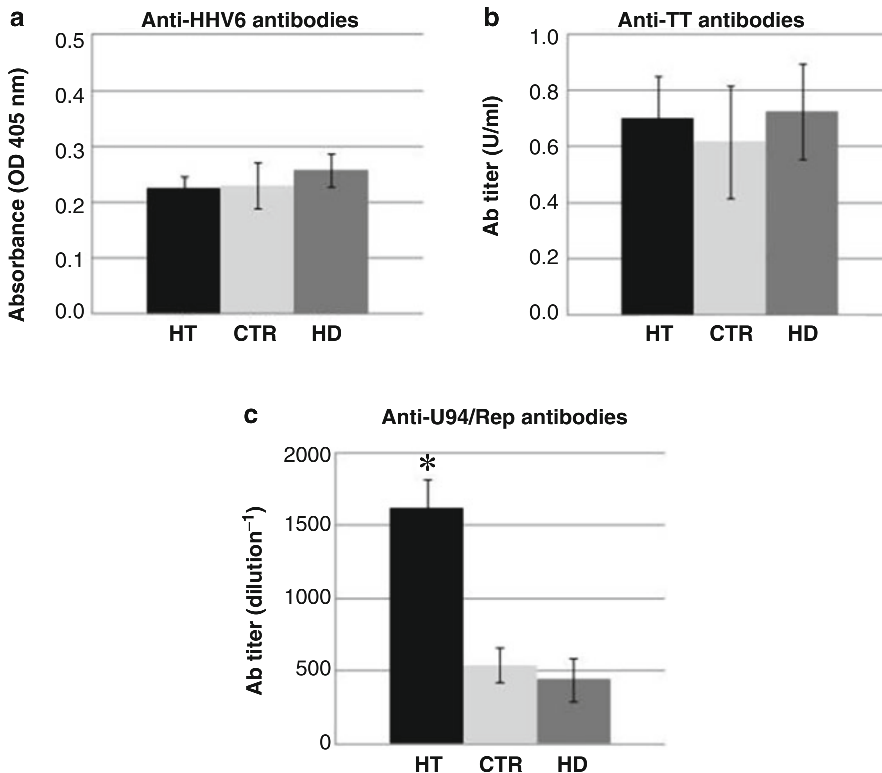

Fig. 1 Anti-HHV6 antibody response in HT and control subjects. Humoral response against (**a**) whole HHV-6 virus, (**b**) Tetanus Toxoid (TT), and (**c**) HHV-6 U94/Rep protein were evaluated by ELISA in Hashimoto's thyroiditis (HT), control (CTR) and healthy donors (HD) groups. Results are expressed as (**a**) mean absorbance at OD_{405nm} ± standard deviation, (**b**) mean Ab titer (U/ml) ± standard deviation, and (**c**) mean Ab titer (dilution^{-1}) ± standard deviation. *$p \leq 0.01$, obtained with two tailed Student *t* test

previously observed different tropism of the two viruses (Caselli et al. 2012a).

The anti-HHV-6 antibody response, evaluated by ELISA using a whole virus lysate (obtained by treatment of purified virions with 0.25 % Triton followed by brief sonication) as the antigen, showed no significant differences in antibody prevalence or titer between HT and control subjects or healthy donors, as well as the anti-tetanus toxin/toxoid (TT) IgG response, used as a control (measured by an ELISA kit, Alpha Diagnostic) (Fig. 1). On the contrary, the antibody

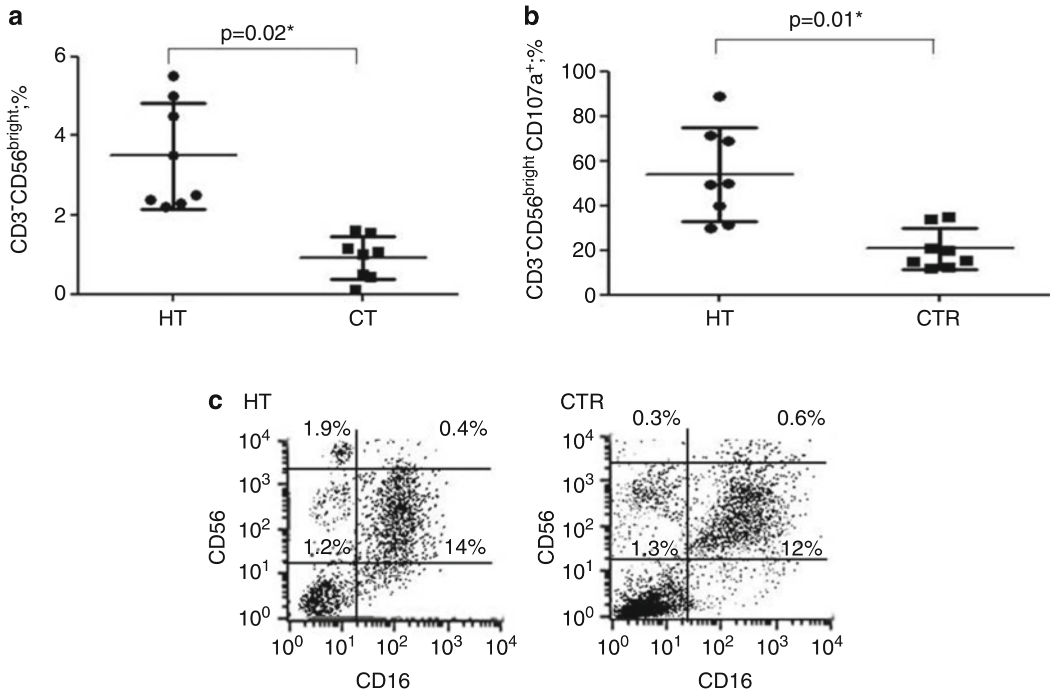

Fig. 2 Cell percentage of (**a**) CD3$^-$CD56^{bright} NK cells and (**b**) CD3$^-$CD56^{bright}CD107a$^+$ in HT and control (CTR) subjects. Data are reported as Mean ± standard deviation. *p value obtained with two tailed Student *t* test. (**c**) Representative dot plots for CD56 and CD16 staining in HT and control (CTR) subjects. Cell percentages are reported

response specifically directed against the HHV-6 U94/Rep protein was more prevalent in HT patients (8/8) than in controls (6/8), and especially the titer was significantly higher in HT *vs* control subjects (1:1624 *vs* 1:543) ($p < 0.01$), who showed prevalence and titer values similar to those of healthy donors (10/12; titer 1:442) (Fig. 1). These results confirmed that HT patients not only have a specific anti-U94/Rep cellular immune response (Caselli et al. 2012a), but also develop specific antibodies against this virus protein.

The analysis of CD3$^-$CD56^{bright} NK cell percentages reported a higher amount of these cells in the samples from HT patients compared with controls ($p = 0.02$; two tailed Student t test) (Fig. 2a). The activation status of CD3$^-$CD56^{bright} NK cells was higher in HT patients compared with controls ($p = 0.01$; two tailed Student t test) (Fig. 2b). On the contrary, CD3$^-$CD56^{dim} NK cells did not present differences in the two groups of subjects (Table 2). Since NK cells are also subdivided into different populations based on the relative expression of CD16, we analyzed the levels of this surface markers. CD16 is a Fc receptor that, upon recognition of antibody-coated cells, delivers a potent signal to NK cells, which eliminate targets through direct killing and cytokine production. When we considered CD16 expression (Fig. 2c, Table 2), we observed that almost all CD3$^-$CD56^{dim} NK cells are CD16+, as previously reported (Poli et al. 2009). On the contrary, there was a slight difference in the percentage of CD3$^-$CD56^{bright}CD16$^-$ NK cells between the two groups (Fig. 2c, Table 2).

When we analyzed the possible association between cell activation of CD3$^-$CD56^{bright} NK cells in HT patients and TPO and Tg Ab levels, we observed a slight correlation between these parameters and CD3$^-$CD56^{bright} NK cell CD107a expression (Fig. 3a, b).

Table 2 Percentages of NK cells in HT and control subjects

	HT patients	CTR	p value
NK cells; %	19.8 ± 5.0	17.0 ± 4.5	0.066*
$CD3^{-}CD56^{dim}$; %	16.9 ± 4.2	15.8 ± 3.7	0.076*
$CD3^{-}CD56^{bright}$; %	2.9 ± 1.9	1.3 ± 0.2	0.02*
$CD56^{-}CD16^{+}$; %	1.5 ± 0.8	1.6 ± 0.6	0.201*
$CD3^{-}CD56^{dim}CD16^{-}$; %	1.2 ± 0.5	1.1 ± 0.7	0.076*
$CD3^{-}CD56^{dim}CD16^{+}$; %	15.4 ± 3.5	14.1 ± 2.8	0.068*
$CD3^{-}CD56^{bright}CD16^{-}$; %	1.6 ± 1.9	0.7 ± 0.13	0.042*
$CD3^{-}CD56^{bright}CD16^{+}$; %	0.6 ± 0.4	0.6 ± 0.1	0.263*
$CD3\text{-}CD56^{+}CD107a^{+}$; %	35.4 ± 13.5	37.6 ± 14.9	0.089*
$CD3\text{-}CD56^{bright}CD107a^{+}$; %	42.0 ± 34.3	24.3 ± 14.1	0.01*
$CD3\text{-}CD56^{dim}CD107a^{+}$; %	10.2 ± 9.6	9.8 ± 8.9	0.052*

*Student *t* test

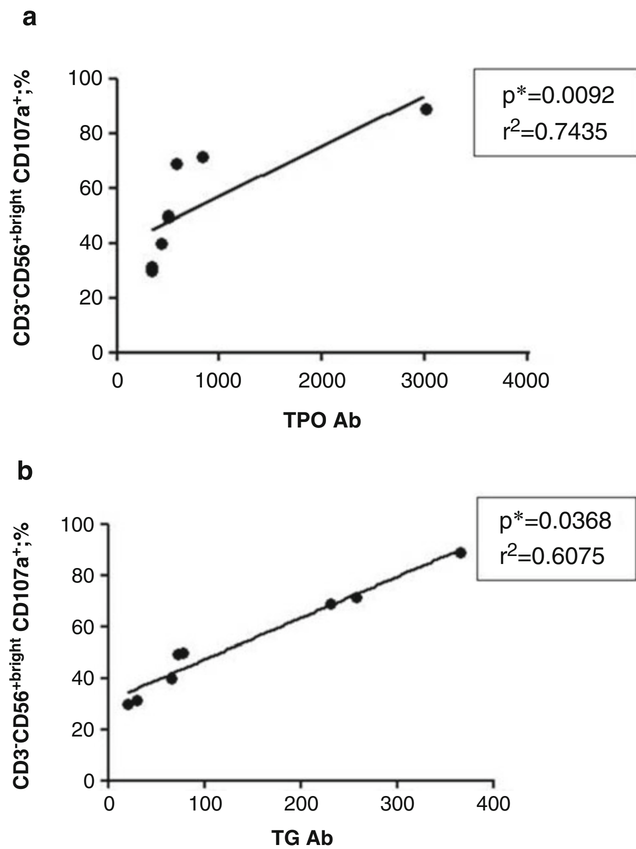

Fig. 3 Correlation between cell activation (CD107a expression) of $CD3^{-}CD56^{dim}$ NK cells from HT patients and disease status, reported as (**a**) TPO and (**b**) Tg Ab levels. *p value obtained with Correlation Z test

These results indicate that NK cells might have an important role for the control of disease activity and viral infection. In fact, we observed an increased NK cell activity in HT patients characterized by HHV-6 infection in FNAs. Previous researches documented the implication of NK cells in the control of both viral infections (Kumagai et al. 2006; Rizzo et al. 2012; Wu et al. 2015) and autoimmune thyroid disease exacerbation (Hidaka et al. 1992). The increase in $CD3^{-}CD56^{bright}$ NK cells, that are characterized by a cytokine-secreting phenotype,

during HHV-6 infection could modify the cytokine environment in HT patients with a possible implication in the disease. In particular, we found an increase in $CD3^{-}CD56^{bright}CD16^{-}$ NK cells, that are known to abundantly produce IFN-γ (Vitale et al. 2004). It is known that cytokines are involved in the pathogenesis of thyroid diseases working in both the immune system and directly targeting the thyroid follicular cells. They are involved in the induction and effector phase of the immune response and inflammation, playing a key role in the pathogenesis of autoimmune thyroid disease. Finally, cytokines can directly damage thyroid cells, leading to functional disorders and may also stimulate the production of nitric oxide and prostaglandin, thus increasing the inflammatory response in HT patients (Mikoś et al. 2014). Moreover, our findings on the increase in $CD56^{bright}$ NK cells in HT patients are in agreement with a previous study that documented the increase in $CD56^{bright}$ NK cells and inflammatory cytokines in the cerebrospinal fluid and serum of a 15-month-old girl with acute necrotizing encephalopathy (ANE) associated with HHV-6 (Kubo et al. 2006).

We are aware that it is difficult to prove etiologic links between viral infections and diseases, especially in the case of a ubiquitous agent such as HHV −6. Moreover, the number of subjects enrolled in this study is limited and a larger cohort is necessary to confirm these results. Nevertheless, our findings indicate that HHV-6 might contribute to HT development, increasing NK cell secretion of inflammatory cytokines sustaining the persistence of an inflammatory status in HT patients.

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