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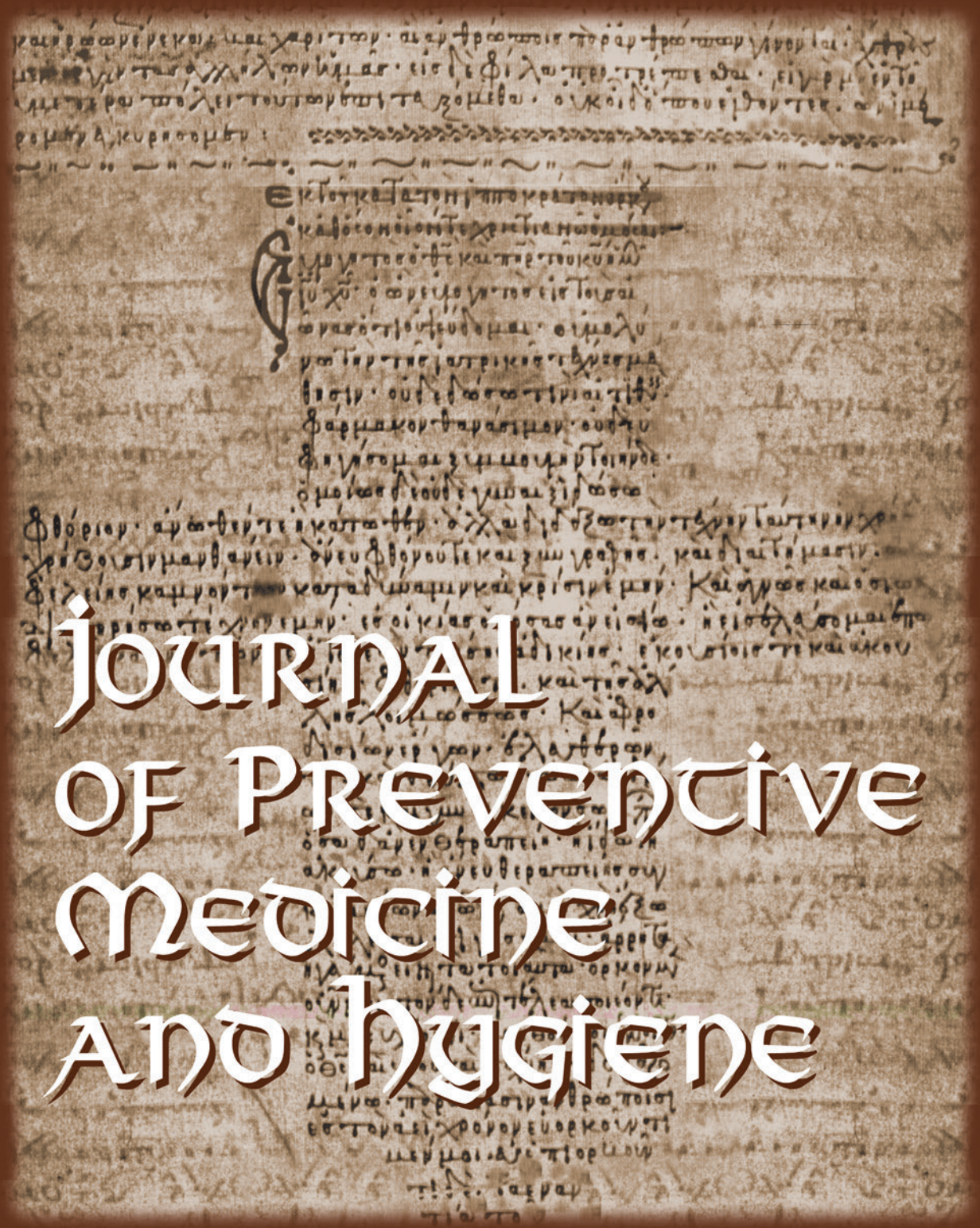

The original document of Hippocrates' oath

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Overview

# Overview of the impact of Typhoid and Paratyphoid fever. Utility of Ty21a vaccine (Vivotif®)

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Keywords

Typhoid fever • Enteric fever • Ty21a vaccine • Vivotif® • *Salmonella typhi* • *Salmonella paratyphi*

Summary

*Cases of diarrhoeal disease number from 1.7 to 5 billion per year worldwide. One of the main causes of diarrhoeal disease is typhoid fever, which is a potentially life-threatening multi-systemic illness. According to the most recent estimates, a total of 26.9 million typhoid fever episodes occurred in 2010. The geographical distribution of the disease differs widely; in developed countries, the incidence rate per 100,000 per year varies from < 0.1 to 0.3, and the disease mainly affects people who travel to endemic areas located in low- and middle-income countries. Low- and middle-income countries are mainly affected owing to the lack of clean water and proper sanitation. In the fight against this plague, prevention is fundamental, and vaccination against typhoid is an effective measure. Vivotif® is an oral live attenuated vaccine which contains a mutated strain of Salmonella (Ty21a) and reproduces the natural infection. The vaccine was first licensed in Europe in 1983 and in the US in 1989, and over the years it has proved efficacious and safe. It is indicated for adults and children from 5 years of age upwards. Specifically, in the most developed countries, vaccination is suggested for high-risk population groups and particularly for international travellers to destinations where the risk of contracting typhoid fever is high. It must also be borne in mind that international travel is increasing. Indeed, international tourist arrivals totalled 1,184 million in 2015 and, on the basis of current trends, international travel is expected to grow by 3-4% in 2017. Vivotif® appears to be a powerful means of disease prevention, the importance of which is highlighted by the spread of antibiotic-resistant strains of Salmonella typhy (S. typhi).*

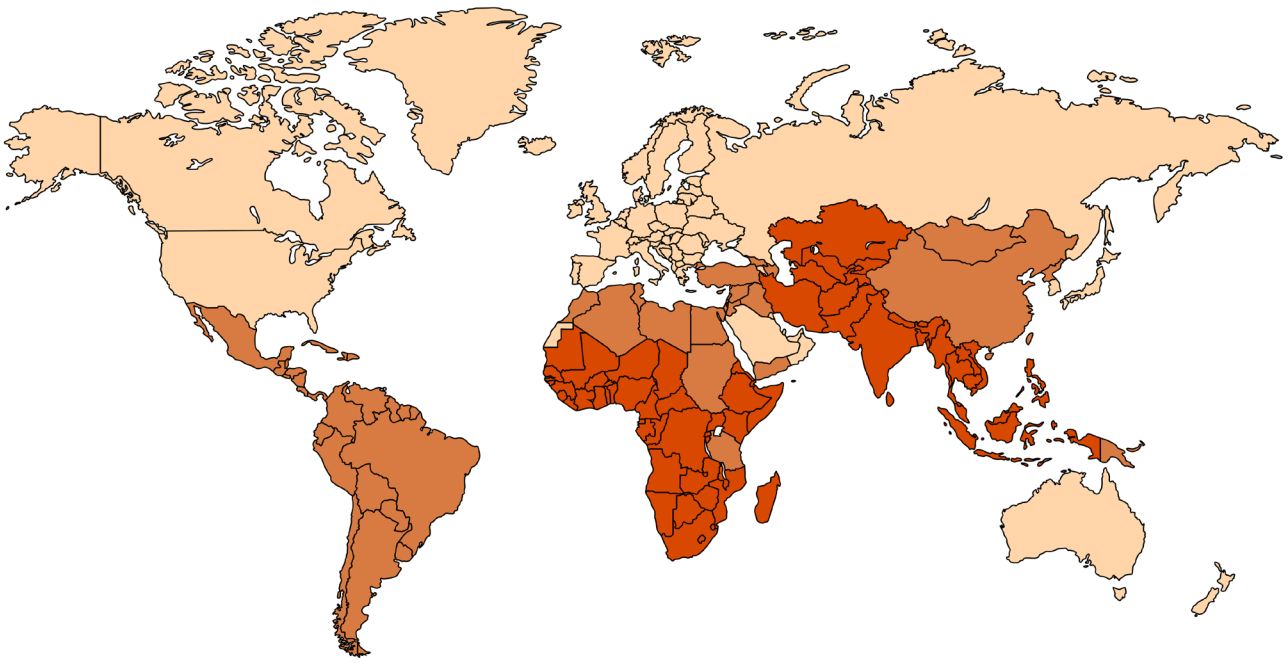
## Introduction

Typhoid Fever (TF), also known as enteric fever, is a potentially life-threatening multi-systemic illness. It is mainly caused by *Salmonella enterica*, subspecies *enterica serovar typhi*, and to a lesser extent by serovars *paratyphi* A, B, and C, which are members of the family of *Enterobacteriaceae* [1]. The genus *Salmonella* is divided into serotypes, on the basis of surface antigens: O antigen based on the lipopolysaccharide component, and H antigen based on flagellar proteins. Moreover, pathogenic strains of *S. typhi* and *S. paratyphi* C present a Vi antigen polysaccharide component [2].
The burden of diarrhoeal disease is very high, accounting for 1.7 to 5 billion cases per year worldwide [3, 4]. The main risk factors of diarrhoeal disease are inadequate drinking water and inadequate sanitation; indeed, in 2014 the World Health Organization (WHO) attributed 502,000 deaths to inadequate drinking water and 280,000 to inadequate sanitation [5, 6]. Furthermore, according to WHO estimates, TF is one of the main causes of foodborne deaths and results in the greatest loss of Disability-Adjusted Life years (DALYs) worldwide [7]. Some authors have called TF "an old plague", asserting that "currently, despite major efforts in preventing and treating cases of enteric fever, millions of new infections (approximately 21 million new cases per year) of typhoid and paratyphoid fevers occur in many areas where poor sanitation and unsafe food and water access occurs frequently and among travellers to endemic areas" [8].
In the fight against this plague, preventive measures are fundamental. Vaccination against typhoid is an effective preventive intervention, especially when coupled with hand-washing, the treatment of household water, and the provision of adequate sanitation [9].
Currently, two well-tolerated and effective vaccines are available. One is based on the use of live attenuated bacteria and is administered orally; the other is based on Vi capsular polysaccharide (Vi-PS), and is administered intramuscularly or subcutaneously [9].
In the present overview, we investigated the epidemiological impact of typhoid and paratyphoid fever and assessed the utility of Ty21a vaccine (Vivotif®) and vaccination policies.

## Epidemiology

As mentioned above, TF is one of the main causes of enteric disease worldwide [10]. The incidence of typhoid fever (overall population) is reported in Figure 1.
According to the most recent estimates, a total of 26.9 million typhoid fever episodes occurred in 2010 [11-14]. The distribution of the disease differs widely through-

**Fig. 1.** Typhoid fever incidence (from Mogasale, et al., mod. [63]).




out the world. In developed countries, the incidence rate per 100,000 per year varies from < 0.1 to 0.3, and the disease mainly affects people who travel to endemic areas located in low- and middle-income countries [11]. Low- and middle-income countries are mainly affected owing to the lack of clean water and of proper sanitation; indeed, the known risk factors for TF are: high population density, unsanitary living conditions, poor hygiene, low socio-economic status, and recent contact with a patient affected by TF [15]. TF has a heavy burden in Asia, with an overall incidence of 170.8 cases per 100,000 people per year, though this estimate varies across the continent [11]. Specifically, Buckle et al. estimated an annual incidence rate of 394.2 per 100,000 in southern Asia. With regard to Africa, the incidence is estimated to be 724.6 cases per 100,000 people per year; however, it is probably underestimated, owing to the lack of information and surveillance systems in the continent [11]. Moreover, Africa suffers many cases of invasive non-typhoid salmonellosis, which are additional confounding factors in estimating the typhoid fever burden [15]. Typhoid fever also affects countries in Latin America, the Caribbean and Oceania, although to a lesser extent, with a median incidence rate of 22.3 cases per 100,000 people per year [14].

The 5-15 year-old age-group is considered the main target of the disease. Notably, even when properly treated, children have a high case fatality rate.

The median FT mortality rate varies from region to region: high-income countries such as North America, Europe, Australia and New Zealand register less than 0.1 death per 100,000 people per year, while the mortality rate is higher in Sub-Saharan Africa (7.2) and Southern Asia (3.9) [11].

*S. paratyphi* causes paratyphoid fever. *S. paratyphi* is thought to cause milder disease than *S. typhi*, with symptoms being predominantly gastrointestinal [16]. While this is probably true of *S. paratyphi* B infection, there are insufficient data to draw conclusions regarding *S. paratyphi* A [17]. In 2000, 5.4 million cases of paratyphoid fever were estimated to have occurred, with incidence rates ranging from 8 cases per 100,000 people per year in high-income countries to 77.4 in low-income countries. Specifically, in Eastern and Southern Asia, the annual incidence is 17.9 cases per 100,000 people. Moreover, *S. paratyphi* A has been found to be responsible for a considerable, and increasing, proportion of cases of enteric fever in some Asian regions [13].

The impact of *S. typhi* and *S. paratyphi* disease is probably underestimated because of inadequate surveillance systems in the most severely affected areas, the low sensitivity of diagnostic tools and healthcare inequalities resulting in low health-seeking behaviour among populations at the highest risk [8].

## Typhoid fever disease

*S. typhi* is restricted to human hosts, and chronic carriers constitute the reservoir of infection.

The disease is mainly transmitted through the consumption of food, drink or water that have been contaminated by the faeces or urine of subjects excreting bacteria (sick or convalescent people or chronic asymptomatic carriers). After *S. typhi* has been ingested, it reaches the intestinal epithelium, where it colonizes macrophages and dendritic cells in the lamina propria; but these fail to destroy the bacterium [9]. Subsequently, bacteria invade the bloodstream, multiply and spread to the lymph nodes, spleen and liver, causing multi-systemic disease [14]. The main manifestations of the disease are fever, which can reach 38°-40°C, and abdominal symptoms (such as diarrhoea or constipation). Nonspecific symptoms, such as weakness, anorexia, headache and dizziness, may precede the fever. Moreover, rose-coloured spots may appear on the trunk, and patients may also experience neuropsychiatric manifestations, hepatomegaly and splenomegaly. The most severe complications are gastrointestinal bleeding, intestinal perforation and typhoid encephalopathy, which occur in 10-15% of patients, generally in the third and fourth weeks of infection [18, 19]. The duration of infection is a major determinant of the risk of severe complications, and a delay in administering appropriate antibiotic treatment may have serious consequences.

Isolation of *S. typhi* from blood is the most common method of diagnosis, though the bacterium can also be isolated from bone marrow, faeces and duodenal fluid. Blood culture displays suboptimal sensitivity, generally being positive in only about 50% of cases. Blood culture also has several limitations, including the volume of blood needed, the need for prompt transport to the laboratory, interference due to prior antibiotic use, limited laboratory expertise and equipment, and expense [13]. Bone marrow culture increases the diagnostic yield to approximately 80% of cases. Stool culture is not usually positive during the earliest phase of the disease [20]. Multiple cultures increase sensitivity and may be required in order to reach a diagnosis.

Although the Widal test is unreliable, it is widely used in developing countries because of its low cost. Newer serologic assays for *S. typhi* infection are occasionally used in outbreak situations, and are somewhat more sensitive and specific than the Widal test; however, they are not an adequate substitute for blood, stool, or bone marrow culture [1].

Early diagnosis and the prompt institution of appropriate antibiotic treatment are essential for the optimal management of TF, especially in children. Ciprofloxacin is commonly used as an empiric treatment, as fluoroquinolones are recommended. However, as fluoroquinolone-resistant or multidrug-resistant strains are spreading, third-generation cephalosporins are used when the possibility of resistance is high [18, 19].

## Preventive measures

In the late 19th and early 20th centuries, typhoid was endemic in all countries, including Europe and the Americas. Subsequently, the widespread use of chlorination, sand filtration, and other means of water treatment drastically reduced the incidence of TF, despite the high prevalence of chronic carriers [14]. Today, TF still places a devastating burden on many low- and middle-income countries; in high-income countries, the impact of the disease is mainly linked to travel to endemic disease areas [16].

## Prevention by vaccines

Currently, 2 typhoid vaccines are internationally available, and both have been shown to be safe and efficacious [9].

The first is an oral vaccine based on a live attenuated *S. typhi* Ty21a strain (Vivotif®), which has been developed in two formulations: enteric coated capsules and a liquid formulation. On the market Vivotif® is available in enteric coated capsules.

The second is a Vi capsular polysaccharide (Vi-PS) vaccine, which is injectable. Furthermore, typhoid conjugate vaccines (TCVs) have been developed, one of which is based on Vi conjugated to rEPA, a recombinant exoprotein A from *Pseudomonas aeruginosa* [22]. Two Vi-tetanus toxoid conjugate vaccines have recently been licensed in India [22].

## Ty21a vaccine (Vivotif®)

Vivotif® is a vaccine which contains a mutated strain of Salmonella (Ty21a) and reproduces the natural infection. The Ty21a strain is a mutant of Ty2 strains lacking Uridine-diphosphate-galactose (UDP-Gal)-4-epimerase. It was obtained in the early 1970s by chemically inactivating the galE gene. Inactivation of the galE gene generates a complete lack of Uridine-diphosphate-galactose (UDP-Gal)-4-epimerase, which is responsible for the conversion of UDP glucose into UDP galactose. As galactose is incorporated into lipopolysaccharide (LPS) as UDP galactose, the lack of galE produces incomplete development of LPS; this results in LPS without the O antigen, which is the chief surface antigen; in this phase, the mutant strain is not immunogenic. However, when the Ty21a strain is alimented by galactose, bacteria are able to generate UDP galactose in an alternative way, expressing a complete and immunogenic LPS. Moreover, owing to the lack of UDP Gal 4-Epimerase, galactose cannot be metabolised and is accumulated in the cytoplasm, resulting in lysis of bacteria and thus eliminating the virulence of the vaccine strain [23-26].

The vaccine was first licensed in Europe in 1983 and in the US in 1989.

The vaccine is administered orally through the ingestion of gastro-resistant capsules (one capsule on days 1, 3 and 5) [27]. It is indicated for adults and children from 5 years of age upwards.

The availability of an oral vaccine constitutes a major step forward in the prevention of typhoid fever, as oral

administration efficaciously stimulates the mechanisms of local, cell-mediated and systemic antibody immunity. Parenteral vaccines lack this triple action.
In countries where the risk of contracting the disease is high, vaccination is recommended every 3 years.
Revaccination every year is recommended if the subject travels from a non-endemic area to an endemic area.
The vaccine can be administered simultaneously with other vaccines and with antimalarial prophylaxis [28].
Vivotif® can be given to HIV-positive subjects who have a CD4 count above 200/mm$^3$.

### Immunogenicity

The immunogenicity of Ty21a vaccine has been evaluated in children and adults in several studies. The assessment of immunogenicity is an important proxy in evaluating efficacy/effectiveness with regard to both the specific strain and paratyphoid fever pathogens.
The vaccine has proved to elicit a good local production of IgA against the O antigen and to induce good humoral and cell-mediated immunogenicity against the O antigen in adult male subjects [29].
The immunogenicity of the Ty21a vaccine was evaluated in 634 Thai children who underwent a three-dose immunization schedule [30]. A seroconversion rate of 60% in 3-year-old and 91% in 6-year-old vaccinated children ($p < 0.005$) was found; this was higher than the seroconversion rate in unvaccinated age-matched children. Seroconversion rates displayed an increasing trend with age in vaccinated children.
Gilman et al. [31] showed that 155 adult males vaccinated with Ty21a vaccine had very good rates of seroconversion of antibodies against the O antigen, resulting in protection from disease.
A study carried out in young Chilean adults (15-19 years old) revealed that serum Ig O antibodies, as assessed by means of ELISA, increased when several doses of the vaccine were administered within a week [32]. In another study, the Ty21a vaccine elicited a strong systemic CD4$^+$ T-helper type 1 response; booster doses induced a significant increase in levels of IgG and IgA anti-LPS in healthy adults [33].
Recently, a study involving volunteers was performed in order to evaluate the immune response against *S. typhi, paratyphyi* A, B and C (cross-reactive). Evaluating specific plasmablasts expressing homing receptors for intestine ($\alpha4\beta7$) demonstrated that the response was great for *S. typhi*, intermediate for *S. paratyphi* B and low for *S. paratyphi* A [34]. In a recent study, Wahid et al. investigated the Ty21a-elicited antibodies which mediate the opsonophagocytosis and intracellular killing of *S. typhi*, *S. parathypi* A and B. The authors found that, after immunization with Ty21a vaccine, opsonophagocytosis increased against *S. typhi* and, in to a lesser degree, also against *S. paratyphi* A and B [35].

### Efficacy and effectiveness

The basic evidence that the Ty21a vaccine protects naïve subjects stems from an experimental study in which adult subjects were challenged with wild *S. typhi* 5-9 weeks after vaccination. Subjects who had received the attenuated strain had a lower attack rate than control subjects (87% efficacy, $p = 0.0002$) [36].
In pre-licensure studies, 4 formulations of the vaccine were evaluated, namely: a lyophilized/bicarbonate formulation "Alexandria formulation", a gelatine capsule/bicarbonate formulation, an enteric-coated capsule formulation, and a sachet "liquid" formulation. Three doses (48 hours apart) of the first formulation were administered to 16,486 Egyptian children (aged 6-7 years) while 3 doses of placebo were administered to 14,557 control group children. Subjects were followed up for three years, during which time 22 cases of typhoid fever were bacteriologically confirmed in the placebo group and one only case was confirmed in vaccine recipients (efficacy 95.6%; CI: 77-99%, $p = 0,001$]) [37]. The gelatine capsule/bicarbonate formulation proved to confer poor protection, and its development was soon abandoned [38].
In a clinical trial performed in Chile on the enteric-coated capsule formulation, a total of 43,759 schoolchildren (aged 6-17 years) received three doses of vaccine. Volunteers were randomly assigned to two groups, one of which received the three capsules within 48 hours (22,179 subjects), and the other within 21 days (21,598). In addition, children (21,906) who received placebo served as the comparator group. Over the 3-year follow-up period, 68 cases of typhoid fever were bacteriologically confirmed in the placebo group, 23 cases were observed in subjects on the short-interval regimen (efficacy 67%; CI: 47-79%, $p < 0.0001$), and 34 cases were confirmed in the long-interval group (efficacy 49%; CI: 24-66%, $p = 0.0006$) [38].
Although the clinical trials of the enteric-coated capsule formulation allowed the Ty21a vaccine to be authorized in several countries, another formulation was tested with the aim of achieving greater efficacy. This formulation, named "sachet liquid formulation", consisted of a vaccine sachet containing lyophilized Ty21a bacteria and another sachet containing a buffer. To formulate the liquid suspension, the contents of the two sachets were mixed in 100 ml of water. In order to study the efficacy of this formulation, two large clinical trials were organized [39, 40]. The first was implemented in Chile and the second in Indonesia. In Santiago, 36,623 schoolchildren were recruited for the administration of three doses of the vaccine, while a comparable group of 10,302 children constituted the placebo control group. Over a 3-year period of follow-up, a significant difference was observed in the incidence of bacteriologically confirmed typhoid fever, which yielded a vaccine efficacy rate of 77% (CI: 60-87%, $p < 0.0001$).
The two above-mentioned formulations of the Ty21a vaccine (enteric-coated capsule and sachet liquid formulation) were compared in a large randomized double-blind trial in Indonesia, in which 20,543 subjects (aged 3-44 years) received 3 doses of either placebo or Ty21a vaccine in enteric-coated capsules or in liquid formulation. All subjects were observed for a follow-up period of 30 months; the rates of blood-culture-positive

typhoid fever recorded were: 810 per 100,000 per year among controls, 379 in the liquid-formulation group, and 468 in the coated-capsule group. Vaccine efficacy was assessed as 53% for the liquid formulation and 42% for capsules [40].

In 2007, Fraser et al. carried out a systematic review and meta-analysis of randomised controlled trials on Typhoid vaccines. Concerning the Ty21a vaccine (3-dose regimen), efficacy was 49% (95% CI: 16-70%), 60% (95% CI: 44-71%), 59% (95% CI: 32-75%), 78% (95% CI: 35-93%) and 47% (95% CI: 24-78%) after 1, 2, 3, 4, and 5 years of follow-up, respectively [41].

With regard to effectiveness, an enlarged study was conducted in Chile on 222,998 subjects, the aims being to verify the possibility of introducing school-based mass vaccination and to ascertain the best management of the vaccination. This investigation demonstrated the good effectiveness of the vaccine and revealed that the regimen of three doses within seven days was the best schedule for the routine vaccination of the target population [42].

The effectiveness studies carried out in Chile provided information on herd immunity effect of Ty21a vaccine; large-scale vaccination appeared to elicit a herd-immunity effect. Two plausible explanations were formulated: first, the excretion of *S. typhi* significantly decreased in vaccinated subjects, causing less environmental contamination; second, the smaller number of temporary carriers reduced *S. typhi* transmission, thereby extending protection to unvaccinated subjects [24].

### Safety and tolerability

The manifold mutations of the Ty21a vaccine make it genetically very stable; indeed, reversion to virulence has not been observed either *in vitro* or *in vivo* [31].

In a study conducted in children (6-7 years) in Alexandria, Wahadan et al. demonstrated the very good safety and tolerability of the Ty21a vaccine [36]. In their controlled trial, the authors recruited 32,388 children (16,486 received the vaccine, 15,902 received oral placebo, and 25,625 did not receive either) and observed that, out of 92,675 doses administered, there were 49 cases of vomiting among vaccinees, versus 21 in the placebo group; 1 case of fever after the vaccine and 3 cases in the control group, and finally 14 cases of abdominal pain in the vaccinated group, versus 2 cases in the placebo group. Obviously, the conclusion was that the vaccine was stable and safe. Furthermore, in the pilot phase of this trial, the Ty21a strain was never detected in the stool for two weeks after vaccine administration [41].

Subsequently, other studies [43-45] demonstrated the good safety of the Ty21a vaccine. Indeed, the study conducted on adults in Chile recorded the following adverse reactions in 385 vaccinees: diarrhoea 1.8%, vomiting 0.5%, fever 0.5%, and rash 0.5%, while in 367 placebo group subjects diarrhoea was found in 1.1%, vomiting in 0.3%, and fever in 0.6% of the subjects. In the study carried out in Indonesia on volunteers of all ages (311 vaccinees with enteric-coated capsule vaccine versus 291 placebo subjects): 3.9% of vaccinees versus 3.1% of placebo subjects had diarrhoea, 1.0% of vaccinees versus 1.7% of placebo subjects suffered from vomiting, 1.7% of vaccinees versus 4.8% of placebo subjects had fever, and 0.3% of vaccinees versus 1.2% of placebo subjects had a rash.

Furthermore, in an extensive field study conducted with the Ty 21a vaccine on 555,000 schoolchildren in Chile [46], passive surveillance did not find vaccine-related adverse effects. Thus, it is now accepted that the Ty21a vaccine is very safe and very well tolerated, and that adverse reactions are rare and self-limiting and consist of: abdominal discomfort, nausea, vomiting, fever, headache, and rash or urticaria [47, 48].

Indeed, in the period 1990-2000, more than 38 million persons received the Ty21a vaccine, with only 743 spontaneous reports of adverse effects, i.e. an incidence of 0.002% [49]. The most common adverse reactions were mild, and mostly temporary, gastrointestinal disorders, followed by general reactions such as fever.

A post-marketing surveillance report published in 2001 mentioned only minor and rare adverse reactions related with the Ty21a vaccine [47].

### Cross-protection against paratyphoid fever

Studies aimed at evaluating the cross-protection of the Ty21a vaccine against paratyphoid fever have been carried out. A study carried out in Area Norte and Occidente of Santiago, Chile, demonstrated protection against paratyphoid B fever (efficacy 49%; 95% CI: 8-73%, $p = 0.019$) [50-54].

There is also some evidence of protection against *S. paratyphi* A infections. Tagliabue et al. found that the Ty21a vaccine induced cellular immunity against *S. paratyphi* A and B, and supposed that the mechanism involved in cellular immunity would be that of antibody-dependent cellular cytotoxicity [51]. A study by Pakkanen et al. demonstrated that the Ty21a vaccine was able to induce the presence of *paratyphi* A plasmablasts in the blood of vaccinees [34]. Furthermore, in 2012, Wahid R. et al. reported that subjects who had received the Ty21a vaccine displayed a humoral immune response of the same magnitude against both *S. typhi* and *S. paratyphi* A [35]. They demonstrated cross-reactive IgA of Antibody Secreting Cells (ASC) responses to *S. paratyphi* A and *S. paratyphi* B LPS following Ty21a vaccination. A subsequent investigation by the same researchers showed that, although the opsonophagocytic antibodies elicited by vaccination were not able to kill *S. paratyphi* A inside the macrophages, phagocytosis of *S. paratyphi* A bacterial cells was increased owing to opsonisation, albeit to a lesser degree than that of B bacterial cells [53]. Subsequently, Wahid R et al. investigated the activity of the Ty 21a vaccine in cross-reactive multifunctional CD8+ T cell responses against *S. typhi*, *S. paratyphi* A and *S. paratyphi* B in humans [54]. They demonstrated that the oral vaccine elicited specific cell-mediated immune responses against *S. typhi* and *S. paratyphi* B, and post-vaccination increases in specific CD8+ T cell responses were observed against all three Salmonella-infected targets. This increase was seen predominantly in the T

Effector/Memory (TEM) cells and in the CD8+CD45RA+ TEM (TEMRA subsets) cells. In another study, these researchers confirmed that live oral typhoid vaccine induced multifunctional *S. typhi*-specific CD4+ T cell responses that cross-react with *S. paratyphi* A and *S. paratyphi* B [54].
These results suggest that the oral live attenuated vaccine elicits protection against *S. paratyphi* A and *S. paratyphi* B. This, albeit modest, degree of protection could, however, yield a milder course of the disease, and may reduce contagiousness. This view is theoretically supported by comparative whole-genome analysis, which shows a high degree of homology among *S. typhi, S. paratyphi* A and *S. paratyphi* B [55, 56].

## Importance of vaccination in the light of *S. typhi* antibiotic resistance

As the antibiotic resistance of *S. typhi* continues to increase, the immunization of subjects at risk appears crucial to containing the spread of typhoid fever. Indeed, since 2001, over the complete genome sequence of Multiple Drug Resistant (MDR) *S. typhi*, the genes of resistance to antibiotics commonly used in the treatment of typhoid fever, and especially to fluoroquinolones, have been identified. Parkill et al. demonstrated that the genes of antibiotic resistance were located in pHCM1 and pHCM2 plasmids and were: the dhfr1b (trimethoprim), su/II (sulphonamide), catI (cholarmphenicol), bla (TEM-1; ampicillin) and strAB (streptomycin) genes [57]. More recently, a study conducted on African isolates suggests that there are at least 3 important MDR lineages (namely: A [haplotypes H56 and, rarely, H42], B [haplotype H55], and C [haplotype H77] [58]. These can be added to the well-known H58 haplotype. The H58 haplotype acquired plasmid-encoding resistance to ampicillin, chloramphenicol and co-trimoxazole, and later acquired resistance to ciprofloxacin because of a chromosomal point mutation [59].
Infection caused by the MDR strains has been documented to be associated with more severe illness and higher rates of complications and death, and with a higher rate of prolonged asymptomatic carrier status [60].

## Conclusions

Enteric fever is a major public health challenge. As the spread of MDR strains of *S. typhi* is increasing, global strategies for combating *S. typhi* infections need to be improved. In this perspective, the most effective way to fight typhoid fever and its severe complications is to improve sanitation, ensure safe supplies of food and water, identify and treat chronic carriers, and implement vaccination. The typhoid vaccine appears to be a powerful means of prevention, and the WHO recommends that countries should consider the programmatic use of typhoid vaccines in order to control endemic disease [60]. A study carried out by Watson et al. has shown that an efficient vaccination programme against typhoid fever can be cost-saving to health services in countries where the disease is endemic; moreover, targeting vaccination to the most seriously affected age-groups would improve cost-effectiveness [61].
The WHO recommends the vaccination of school-age and/or preschool-age children in areas where typhoid fever in these age-groups is known to be a significant public health problem, particularly where antibiotic-resistant *S. typhi* is prevalent, and during outbreaks [60]. In the most developed countries, vaccination is suggested for high-risk population groups (such as persons with intimate exposure to chronic carriers, microbiologists and other laboratory workers), and particularly for international travellers to destinations where the risk of typhoid fever is high and/or in locations where antibiotic-resistant strains of *S. typhi* are prevalent. It must also be borne in mind that international travel is increasing. Indeed, in 2010, the number of international tourist arrivals worldwide reached 949 million, and a total of 1,184 million was registered in 2015, according to the latest UNWTO World Tourism Barometer [62]. On the basis of current trends, international travel is expected to grow by 3-4% in 2017.
In conclusion, Vivotif® appears to be a powerful means of preventing enteric fever. Indeed, over the years it has proved efficacious, eliciting a triple immunologic response. Furthermore, clinical studies have also demonstrated its partial cross-protection against *S. paratyphi* and large-scale vaccination has appeared to elicit a herd-immunity effect. The vaccine is very safe and well tolerated, and its oral administration ensures very good compliance.

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The authors declare no conflict of interest.
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## Authors' contribution

RG and DP conceived and designed the overview. DA, LA and FZ performed a search of the literature on epide-miology of typhoid and paratyphoid disease. RG carried out a search of literature on the immunogenicity, effi-cacy and safety of the oral live attenuated vaccine. All authors contributed to the draft of the article. RG, DA and DP revised critically the manuscript. RG supervised the manuscript. All authors read and approved the final version of the manuscript.

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Overview

# The history of tuberculosis: from the first historical records to the isolation of Koch's bacillus

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Keywords

Tuberculosis • History • Koch's bacillus

Summary

*Tuberculosis (TB) is a contagious, infectious disease, due to Mycobacterium tuberculosis (MT) that has always been a permanent challenge over the course of human history, because of its severe social implications. It has been hypothesized that the genus Mycobacterium originated more than 150 million years ago. In the Middle Ages, scrofula, a disease affecting cervical lymph nodes, was described as a new clinical form of TB. The illness was known in England and France as "king's evil", and it was widely believed that persons affected could heal after a royal touch. In 1720, for the first time, the infectious origin of TB was conjectured by the English physician Benjamin Marten, while the first successful remedy against TB was the introduction of the sanatorium cure. The famous scientist Robert Koch was able to isolate the tubercle bacillus and presented this extraordinary result to the society of Physiology in Berlin on 24 March 1882. In the decades following this discovery, the Pirquet and Mantoux tuberculin skin tests, Albert Calmette and Camille Guérin BCG vaccine, Selman Waksman streptomycin and other anti-tuberculous drugs were developed.*

## Introduction

Tuberculosis (TB) is a contagious, infectious disease, due to *Mycobacterium tuberculosis* (MT), which usually lasts throughout the life course and determines the formation of tubercles in different parts of the body [1]. MT has very ancient origins: it has survived over 70,000 years and it currently infects nearly 2 billion people worldwide [2]; with around 10.4 million new cases of TB each year, almost one third of the world's population are carriers of the TB bacillus and are at risk for developing active disease [3].
TB has always been associated with a high mortality rate over the centuries, and also nowadays, it is estimated to be responsible for 1.4 million TB deaths, among infectious diseases after human immunodeficiency virus (HIV) [3].
Due to its infectious nature, complex immunological response, chronic progression and the need for long-term treatment, TB has always been a major health burden; in more recent years, the appearance of multi-drug resistant forms and the current TB-HIV epidemic, associated with its severe social implications, treating and preventing TB have represented a permanent challenge over the course of human history [4, 5].

## Ancient times: the first historical records

It has been hypothesized that the genus *Mycobacterium* originated more than 150 million years ago. *Mycobacterium ulcerans,* causing infections since ancient times, requires specific environmental conditions as reflected nowadays in its distribution worldwide [6].
Three million years ago, an early progenitor of MT might have infected early hominids in East Africa [7] and 20.000-15.000 years ago, for the first time, the common ancestor of modern strains of MT might have appeared [8, 9].
Egyptian mummies, dating back to 2400 BC, reveal skeletal deformities typical of tuberculosis; characteristic Pott's lesions are reported and similar abnormalities are clearly illustrated in early Egyptian art [10, 11].
Nevertheless, no evidence about TB lesions is reported in Egyptian papyri. The first written documents describing TB, dating back to 3300 and 2300 years ago, were found in India and in China respectively [12, 13].
Other written documents connected to TB are related to the Hebraism. The ancient Hebrew word *schachepheth* is used in the Biblical books of Deuteronomy and Leviticus in order to describe TB [14]; in the same period, in the Andean region, archeological evidence of early TB, including Pott's deformities, was provided by Peruvian mummies, suggesting that the disease was present even before the colonization of the first European pioneers in South America [15-18].
In the Ancient Greece TB was well known and called Phtisis. Hippocrates described Phtisis as a fatal disease especially for young adults, accurately defining its symptoms and the characteristic tubercular lung lesions.

Excellent discoveries of the early scientists who studied TB were made in the same period: in Greece, Isocrates was the first author supposing that TB was an infectious disease, while Aristotle suggested the contagious nature of "king's evil" in pigs and oxes [19].
In Roman times, TB is mentioned by Celso, Aretaeus of Cappadocia and Caelius Aurelianus, but it is not recognized as sharing the same etiology of extrapulmonary manifestations such as scrofula, Pott's disease and TB lupus.
According to the Greek Clarissimus Galen, who became personal physician of the Roman Emperor Marcus Aurelius in 174 AD, the symptoms of TB include fever, sweating, coughing and blood stained sputum; he recommended fresh air, milk and sea voyages as successful treatments for the disease [20-22].
After the decline of the Roman Empire, TB was widespread in Europe in the VIII and XIX centuries, as witnessed by several archaeological findings [23].
The Byzantine doctors Aetius of Amida, Alexander of Tralles and Paul of Aegina described the pulmonary and glandular forms of TB [24], while in the Arabic Empire, Avicenna supposed the contagious nature of TB.

## Middle Ages and Renaissance time: the "king's evil" and the discovery of extra pulmonary TB

In the Middle Ages, scrofula, a disease affecting cervical lymph nodes, was described as a new clinical form of TB. The illness was known in England and France as "king's evil", and it was widely believed that persons affected could heal after a royal touch [25].
In the 12th century, William of Malmesbury reported complementary treatments including visits at royal tombs, the kings' touch or the use of a coin-talisman.
The practice of the king's touch established by English and French kings continued for several years. Queen Anne was the last English monarch to use this practice (1712), George I put an end to it in 1714, while in France it continued up to 1825 [26].
In the Middle Ages, moreover, the French surgeon Guy de Chauliac (1363) for the first time proposed a healing intervention for the cure of the "king's evil" [27].
Guy de Chauliac was also strongly in favour of the removal of scrofulous gland with an engraving, as recommended by Paul of Aegina, who advised the surgical removal of the diseased gland, taking care not to harm vessels or nerves of which the neck region is rich [28].
As far as concerns the contagious nature of TB, a clear definition was first given by Girolamo Fracastoro in the sixteenth century [29].
The exact pathological and anatomical description of the disease was illustrated in 1679 by Francis Sylvius, in his work *Opera Medica*, in which he describes tubercles, their progression to abscesses, cavities and empyema in the lungs and in other sites of consumptive patients [30].
Short afterwards, in Italian health law, in particular in an edict issued by the Republic of Lucca in 1699, there is the first official reference to the infectious nature of the disease [31]. In 1735 the Health Board of the Republic ordered the compulsory notification and isolation of consumptives, forbidding their admission in public hospitals, and establishing specific places for their treatment [29].

## XVIII-XIX centuries: the infectious theory and the isolation of the Koch bacillus

In 1720, for the first time, the infectious origin of TB was conjectured by the English physician Benjamin Marten, in his publication "A new theory of Consumption". For the early eighteenth century, Marten's writings display a great degree of epidemiological insight [32].
Both terms consumption and phthisis were used in the 17th and 18th centuries, until in the mid-19th century Johann Lukas Schönlein coined the term "tuberculosis" [33].
In the 18th century in Western Europe, TB had become epidemic with a mortality rate as high as 900 deaths per 100,000 inhabitants per year, more elevated among young people. For this reason, TB was also called "the robber of youth".
During the industrial revolution, the diffusion of particularly problematic social conditions, such as extremely deprived work settings, poorly ventilated and overcrowded housing, primitive sanitation, malnutrition and other risk factors, were intimately associated with the disease [26].
In 1838-39, up to a third of English tradesmen and employees died of TB, whereas the same proportion decreased to a sixth in the upper class [34].
The extreme anemic pallor of people affected by TB was at the origin of the new term "white plague", coined during the 18th century [20, 35].
One hundred years later, TB was defined as "Captain of All These Men of Death" because of its epidemic proportions in Europe and North America, determining one in four deaths.
At the beginning of the 19th century, there was a large scientific debate about different theories concerning the etiopathological origin of phthisis, arguing whether it might be considered: an infectious disease – as generally considered in Southern Europe – an hereditary one – as stated in Northern Europe – or a form of cancer. On the other hand, the discussion was about scrofula, tubercles, and phthisis as separate disease entities or manifestations of the same illness [26].
In 1793, the caseous necrosis, "cheese-like", phthisic abscesses were named "tubercles" by the Scottish pathologist Matthew Baille [36].
In 1810, the French physician Gaspard-Laurent Bayle of Vernet described the disseminated "miliary" TB in his work *Recerches sur la phthisie pulmonaire*, recognizing TB not only as a disease affecting the lung, but a generalized one, clinically defined by coughing, difficulty in breathing, fever and purulent expectoration [37, 38].
In 1819, the French Theophile Laennac identified the presence of consolidation, pleurisy and pulmonary cavitation as pathognomonic signs of pulmonary or extrapulmonary TB [33]. *Mycobacterium tuberculosis* most commonly affects the respiratory tract, but it could also

infect gastrointestinal, bones, joints, nervous systems, lymph nodes, genitourinary tract and skin with inflammatory infiltration, caseation, necrosis, abscesses, fibrosis, formation of tubercles and calcification [39, 40].
He recognized tubercles as the characteristic signs of the first phase of phthisis. He described their first appearance in the lungs, in their "miliary" ("millet seed-like") form, their progressing to larger tubercles containing "cheese-like" ("caseous") material, their breakdown into pus, and eventually forming cavities and empyema.
Extra-pulmonary phthisic tubercles were recognized in the intestines, liver, meninges and other organs, as also described by Sir Percivall Pott, a British surgeon that in 1779 defined as "Pott's disease" the vertebral collapse and spinal cord paralysis caused by TB infection [33, 36, 37, 41, 42].
In 1843, the German physician Philipp Friedrich Hermann Klencke succeeded in the experimental reproduction of human and bovine forms of TB, causing generalized TB in rabbits, through a successful inoculation of material from a miliary tubercle into their liver and lungs [27].
In 1849 Lebert, publishing his work *Traite Pratique des Maladies Scrofuleuses et Tuberculeuses*, suggested that the "King's evil" was a childhood disease that might cause suppuration and ulceration of different body's sites such as skin, ears, eyes, joints, bones, with a different pathogenesis from TB [43].
The first successful remedy against TB was the introduction of the sanatorium cure, described for the first time in 1854 in the doctoral dissertation "Tuberculosis is a curable disease" by Hermann Brehmer, a botany student suffering himself from TB, who reported his healing after a travel to the Himalayan Mountains [44].
Afterwards Brehmer founded an institution in Gorbersdorf, a mountain town situated in a fir forest, in order to cure patients with continuous fresh air and good nutrition. The subsequent sanatoria were built with the same setup and permitted to cure a lot of TB patients in the next decades [45].
The infectious nature of TB was demonstrated in 1865 by Jean-Antoine Villemin, a French military surgeon at the Army Medical School.
He formulated his hypothesis observing that TB was more frequent among soldiers who stationed for long times in barracks than among those in the field.
He also highlighted how healthy army recruits coming from the countryside often became consumptive some months after the beginning of their service.
Villemin's experiments consisted in inoculating a rabbit with "a small amount of purulent liquid from a tuberculous cavity" removed at autopsy from an individual died of TB [20].
As described in Villemin's work *Cause et nature de la tuberculose: son inoculation de l'homme au lapin,* the inoculated animal remained alive and no disease signs were discovered, but at autopsy, three months later, extensive TB was evident [46].
Villemin suggested that phthisis could be similar to glanders, an infectious disease in horses [20, 34, 36, 37, 47].
The infectious theory was well documented in Villemin's work *Études sur la tuberculose. Preuves rationelles et expérimentales de sa spécificité et de son inoculabilité*, dated 1868 [48], in which he stated the presence of TB-like illnesses in different animals [49].
The author also noticed that more crowded urban areas had a higher prevalence of TB and that some parts of the world, like New Zealand and Australia, seemed to have not known TB until the arrival of pioneers.
Some years later, in 1867, Theodor Albrecht Edwin Klebs was one of the early scientists to try to isolate the TB bacillus, sowing tuberculous material on egg white, stored in sterile flasks.
In his experiments, the culture was quickly muddy and it was possible to recognize mobile bacilli, which, after inoculation into the peritoneal cavity, caused the disease in Guinea pigs [50].
The famous scientist Robert Koch was able to isolate the tubercle bacillus. Using the methylene blue staining recommended by Paul Ehrlich, he identified, isolated and cultivated the bacillus in animal serum. Finally he reproduced the disease by inoculating the bacillus into laboratory animals [51].
Robert Koch presented this extraordinary result to the Society of Physiology in Berlin on 24 March 1882, determining a milestone in the fight against TB [52].
In the decades following this discovery, the Pirquet and Mantoux tuberculin skin tests, Albert Calmette and Camille Guérin (BCG) vaccine, Selman Waksman streptomycin and other anti-tuberculous drugs were developed. Koch contributed also to the elucidation of the infectious etiology of TB and for his scientific results, he was awarded the Nobel prize in Medicine in 1905 [33, 51].
Nowadays TB is still a major public health problem, for this reason a combined strategy, based on improving drug treatment, diagnostic instruments, and prevention strategy, is necessary, in order to eradicate *M. Tuberculosis* by the year 2050, as committed by the World Health Organization (WHO) [53].

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## Authors' contribution

MM conceived and designed the overview. IB and LG performed a search of the literature and contributed to the draft of the article. NLB revised critically the manuscript. MM supervised the manuscript. All authors read and approved the final version of the manuscript.

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ORIGINAL ARTICLE

# Frequently asked questions on seven rare adverse events following immunization

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Keywords

Immunization • Adverse events • Thrombocytopenia • Guillain-Barré syndrome

Summary

*Routine mass immunization programs have contributed greatly to the control of infectious diseases and to the improvement of the health of populations. Over the last decades, the rise of anti-vaccination movements has threatened the advances made in this field to the point that vaccination coverage rates have decreased and outbreaks of vaccine-preventable diseases have resurfaced. One of the critical points of the immunization debate revolves around the level of risk attributable to vaccination, namely the possibility of experiencing serious and possibly irreversible adverse events. Unfortunately, the knowledge about adverse events, especially rare ones, is usually incomplete at best and the attribution of a causal relationship with vaccinations is subject to significant uncertainties. The aim of this paper is to provide a narrative review of seven rare or very rare adverse events: hypotonic hyporesponsive episode, multiple sclerosis, apnea in preterm newborns, Guillain-Barré syndrome, vasculitides, arthritis/arthralgia, immune thrombocytopenic purpura. We have selected these adverse events based on our experience of questions asked by health care workers involved in vaccination services. Information on the chosen adverse events was retrieved from Medline using appropriate search terms. The review is in the form of questions and answers for each adverse event, with a view to providing useful and actionable concepts while not ignoring the uncertainties that remain. We also highlight in the conclusion possible future improvements to adverse event detection and assessment that could help identify individuals at higher risk against the probable future backdrop of ever-greater abandonment of compulsory vaccination policies.*

## Introduction

In the last 10 years, great advances have been made in developing and introducing new vaccines and expanding immunization programs. More people than ever before are being vaccinated and access and use of vaccines by age groups other than infants is expanding. As a result of immunization combined with other health care and development interventions – including improved access to clean water and sanitation, better hygiene and education – the annual number of deaths among children under five years of age fell from an estimated 9.6 million in 2000 to 7.6 million in 2010, despite an increase in the number of children born each year [1].

According to World Health Organization (WHO) data, vaccination prevents 2-3 million deaths every year worldwide. At the same time, WHO warns that globally 22 million newborns do not receive basic immunization; among them there are 700,000 newborns belonging to the WHO European Region. Vaccination is perceived as unsafe and unnecessary by a growing number of parents in large part because diseases that were once the cause of many outbreaks and loss of health and life are now rarely seen, because they have been prevented by vaccines. In this perspective, a rare potential for vaccine-induced harm can loom large when people no longer experience the targeted disease, a case of vaccines being the victims of their own success. In fact anti-vaccination movements have been implicated in lowered vaccine acceptance rates and in the increase in vaccine-preventable disease outbreaks and epidemics. In large part, this is caused by mistrust in the medical system and by fear of adverse events (AEs) that doctors are unwilling to talk about. On the contrary, research has shown that exposure to scientific proof can be effective in boosting patients' knowledge; in the USA, the Centers for Disease Control and Prevention (CDC) published information aiming to educate the reader and dispel the most widespread myths [2-5].

In spite of the fact that vaccines currently used in immunization programs are safe, like all medicinal products they have potential health risks and can cause adverse events, defined as harmful and unintended effects following the use of a medicinal substance [6].

Regarding vaccines, an Adverse Event Following Immunization (AEFI) is any harmful clinical event that occurs after vaccine administration and that does not necessarily have a causal relationship with vaccine use.

In this context an AEFI could be an unintentional unfavorable sign, a laboratory test anomaly, a symptom or a disease [7].
It is important to point out that in this definition of AEFI, only a temporal association is considered and no causal relationship is implied; indeed, much of the assessment of AEFIs is devoted to ascertaining whether a causal relationship actually exists (under different degrees of likelihood, i.e. certainly, probably, possibly related or unrelated).
The scientific knowledge about AEFIs is limited because their notification is frequently overlooked by physicians (under-notification) and because the available adverse event data frequently show only temporal relationships between vaccination and the onset of an AEFI. Indeed the establishment of causality is challenging and requires much time and labor. In fact, the largest review of adverse events to date, published in 2012 by the Institute of Medicine of the USA (IOM), explains in the Preface that in the majority of cases, available scientific information was not sufficient to conclude whether a particular vaccine caused a specific rare AEFI [8, 9]. One of the reasons is that even very large epidemiologic studies may not be able to detect or rule out rare (or very rare) AEFIs. An AEFI is conventionally considered rare if it occurs in < 1/1000 but > 1/10000 individuals (very rare if < 1/10000 individuals). There is a residual category of AEFI for which frequency is unknown because they were spontaneously reported after the vaccine was marketed and therefore the denominator required to compute frequency was unavailable [10].
There are many elements to be considered in assessing the risk-benefit balance of vaccine administration. First of all, the advantages of vaccine administration have to be taken into account, in terms of benefits (protection) afforded by each dose administered.
Next, the likelihood of contracting the disease must be considered, which is itself related to the incidence of the disease, to the vaccine coverage rate in the population and to other factors that can increase risk, for example exposure to a case, occupational risk or travels to highly endemic areas.
A thorough assessment of the frequency, severity, and causal relation of AEs (and their complications) is also necessary, especially for Serious Adverse Events (SAEs) [11]. A SAE is any adverse event that either results in death, or is life-threatening, or requires inpatient hospitalization or causes prolongation of existing hospitalization, or results in persistent or significant disability/incapacity, or is a congenital anomaly/birth defect, or requires intervention to prevent permanent impairment or damage [12].
The need to answer questions from patients and health care workers (HCWs) and to choose whether to vaccinate an individual or not, based on a risk-benefit assessment that takes into account the susceptibility of the individual (a form of personalized medicine), forms the basis of our review that aims to provide actionable answers to doubts about a selection of rare, very rare or of unknown frequency adverse events.

We focused on the issues regarding risk assessment, risk communication and risk management for seven AEFIs:
- Hypotonic Hyporesponsive Episode (HHE);
- Multiple Sclerosis (MS);
- Apnea in the pre-term newborn (APTN);
- Guillain-Barré Syndrome (GBS);
- Vasculitides;
- Arthritis/Arthralgia (AA);
- Immune Thrombocytopenic Purpura (ITP).

## Methods and search results

We performed a literature search on Medline using PubMed up to August 26th 2016. We applied neither language nor date of publication restrictions and we performed the search using the following search terms individually and in combination: vaccin*; immuniz*; immunis*; thrombocytopenia; apnea; hypotonic hyporesponsive; Guillain Barré; multiple sclerosis; arthritis; arthralgia; vasculitis.

**Tab. I.** Search strategy and results.

| Search item | Search terms (S) | Number retrieved (Numbers in brackets used in combined searches) |
|---|---|---|
| S1 | Vaccin* | (323853) |
| S2 | Immuniz* | (165606) |
| S3 | Immunis* | (11117) |
| S4 | Thrombocytopenia | (64342) |
| S5 | Apnea | (46708) |
| S6 | Hypotonic hyporesponsive | 47 |
| S7 | Guillain Barré | (8165) |
| S8 | Multiple Sclerosis | (70531) |
| S9 | Arthritis | (281729) |
| S10 | Arthralgia | (13873) |
| S11 | Vasculitis | (97428) |
| S12 | S1 AND S4 | 644 |
| S13 | S1 AND S5 | 125 |
| S14 | S1 AND S7 | 615 |
| S15 | S1 AND S8 | 999 |
| S16 | S1 AND S9 | 2057 |
| S17 | S1 AND S10 | 223 |
| S18 | S1 AND S11 | 533 |
| S19 | S2 AND S4 | 878 |
| S20 | S2 AND S5 | 82 |
| S21 | S2 AND S7 | 345 |
| S22 | S2 AND S8 | 1333 |
| S23 | S2 AND S9 | 2791 |
| S24 | S2 AND S10 | 63 |
| S25 | S2 AND S11 | 433 |
| S26 | S3 AND S4 | 42 |
| S27 | S3 AND S5 | 20 |
| S28 | S3 AND S7 | 33 |
| S29 | S3 AND S8 | 59 |
| S30 | S3 AND S9 | 184 |
| S31 | S3 AND S10 | 9 |
| S32 | S3 AND S11 | 19 |

We also used the report of the Institute of Medicine [8] for reference.

We have chosen to answer for each AEFI the following frequently asked questions (FAQs) based on the requests for information that HCWs routinely ask:

- definition and incidence of the adverse event;
- biological plausibility based on the existence of pathophysiological mechanisms that lead to adverse event onset, and causality assessment;
- risk factors for AEFI onset after vaccination;
- types of vaccines possibly associated with the adverse event and AE management;
- special considerations in vaccine administration to patients:
  - who experienced the AEFI following a previous immunization.
  - who suffer from or have had episodes of the disease in question (In this context we use the term "disease" when the AEFI corresponds to a well-recognized disease and we consider the disease to be a possible risk factor for its own exacerbation after immunization).

For the AEFI, we used validated case definitions where available and, as regards pathophysiology, we reported the main hypotheses according to current scientific knowledge. Selection of information on the vaccines potentially implicated in the various AEFIs, on the main risk factors and on the recommendations for immunization of individuals who previously experienced the AEFI, was also performed based on the reports available from the literature. The results obtained from the search strategy are shown in Table I.

## Frequently Asked Questions (FAQs)

### Hypotonic Hyporesponsive Episode (HHE)

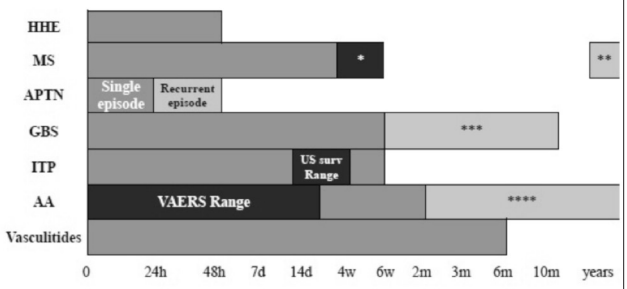
**How is a case of HHE defined and how frequent is it?**

HHE is a clinical condition which has the following features: reduced muscle tone, hyporesponsiveness and change of skin color, in the absence of cardiologic (particularly of heart rhythm), electroencephalographic and glycemic alterations [13]. Apart from the clinical triad of signs there are no further investigations helpful in confirming the diagnosis of HHE [14-16]. The reported rates following a whole cell pertussis vaccine ranges from 0 - 291 per 100,000 doses [17, 18]. The median time to onset of signs after immunization is 3–4 h but ranges from immediately to 48 h post-immunization, while the median duration of the triad signs is 6–30 min [16] (Fig. 1).

**What are the pathophysiological mechanisms underlying HHE?**

Pathophysiology of HHE is currently unknown. It was hypothesized in the first studies about this AE, that the pathophysiological mechanism could be an exaggerated vagal response, but the timing of onset of HHE and the lack of ECG, EEG and glycemic alterations led to refuting this hypothesis and, at the present time, no new hypotheses have been put forward. The main efforts have been put into defining correctly HHE in order to avoid mistakes in diagnosis and notification [16, 19, 20].

**Fig. 1.** Time frame of attribution of selected AEs to vaccination.




Legend:

: Time frame of AE onset during which the AE is commonly attributed to vaccination

HHE: Hypotonic Hyporesponsive Episode [16].
MS: Multiple Sclerosis; Dark grey box [27];
Black box: * trend identified only in a small sample of cases [27];
Light grey box: ** trend identified by some authors but not confirmed [28].
APTN: Apnea in Pre-Term Newborns; Dark grey box: single episode [34-37,42,43];
Light grey box: recurrent episode [41,52]
GBS: Guillain-Barré Syndrome; Dark grey box [28,67,78];
Light grey box: *** extension of time window of onset suggested by some authors [60].
ITP: Immune Thrombocytopenic Purpura; Dark grey box [127];
Black box: range identified by US postmarketing surveillance [132].
AA: Arthralgia and Arthritis; Black box: range identified by VAERS [104];
Dark grey box: [105];
Light grey box: **** extension of time window of onset suggested by some authors [90].
Vasculitides: Dark grey box [84,92].

**Which are the vaccines associated with HHE onset?**

The first cases were reported in children under 5 years of age following administration of whole-cell pertussis vaccine [15, 16]. Over the last 20 years, cases of HHE have been reported following administration of diphtheria, tetanus, *H. influenzae B*, Hepatitis B Virus, 13-valent pneumococcal, acellular pertussis vaccines. In the latter case, the frequency appears to be lower than with the whole-cell vaccine [13, 16, 21].

**What are the risk factors for HHE and how can it be managed?**

No risk factors have been identified and so there are no specific precautions to be taken.

According to studies based on the follow-up of HHE relying on parental reporting and neurodevelopmental testing, HHE is a self-limiting event without long-term sequelae [16], so it is important to reassure the parents that it is an event that does not lead to complications.

**What is the recommended action regarding immunization in case of a previous episode of HHE?**

According to a recent Polish study, in a group of 49 children who experienced HHE following diphtheria-tetanus-whole cell pertussis vaccination (DTwP), 2 children experienced a second episode of HHE after a second consecutive dose of DTwP [22]. According to this study, it seems that HHE can recur more frequently in children who have already experienced it compared to the general population. In spite of this observation, no contraindications to subsequent vaccination exist for these children.

### Multiple Sclerosis (MS)

**How is a case of MS defined and how frequent is it?**

Multiple Sclerosis (MS) is an acquired chronic immune-mediated inflammatory condition of the central nervous system (CNS), affecting both the brain and spinal cord. It is the most frequent cause of serious physical disability in working age people. The person affected by MS typically develops symptoms in their late 20s, experiencing visual and sensory disturbances, limb weakness, gait problems and symptoms which affect urinary and gastrointestinal tracts. The clinical course is often characterized at the beginning by a partial recovery, but over time MS tends to evolve toward increasing disability [23]. In 2007, globally, the median estimated prevalence of MS was 30 per 100,000 while regionally, the median estimated prevalence of MS was greatest in Europe (80 per 100,000), followed by the Eastern Mediterranean (14.9), the Americas (8.3), the Western Pacific (5), South-East Asia (2.8) and Africa (0.3). The total estimated number of people diagnosed with MS was approximately 1.3 million [24].

**What are the pathophysiological mechanisms underlying post-vaccination MS?**

Pathophysiology of post-vaccination MS is uncertain. The first hypothesis was based on the discovery, in 1985, of a molecular mimicry phenomenon between Hepatitis B Virus (HBV) polymerase and myelin basic protein, but in light of subsequent research, today this association appears to be far less significant [25, 26]. Currently the most plausible mechanism seems to be hyperstimulation of the immune system that acts as an enhancer of a pre-existing autoimmune vulnerability [27].

**Which are the vaccines associated with MS onset?**

According to a case-control study in France in a pediatric population, it was observed that in the three years following HBV vaccination the odds for developing an MS flare were 1.5 but this result was not statistically (95%CI) significant [28]. In a subsequent case-control study in California, a possible increase in central demyelination risk in the first 30 days after any vaccination was reported in subjects under 50 years of age. A trend towards an association of MS and any type of vaccination within 42 days (6 weeks) after immunization was identified (OR: 2.32; 95%CI: 1.18-4.57), but this result was obtained in a small sample of cases and cannot be generalized at the present time (Fig. 1). Moreover, since this association has not been observed in the longer term in the above study, it is hypothesized that vaccination could act as a trigger for underlying MS [27].

**Which are the risk factors for MS and how do they affect immunization?**

There are several risk factors (15-60 years of age, female gender, family history, Epstein Barr Virus infection, etc.) for MS but they do not affect routine immunization practice since the association between MS and vaccination has not been demonstrated in spite of numerous studies on this topic [8].

**What is the recommended action regarding immunization in case of previous or ongoing acute episodes of MS?**

Inactivated vaccines are generally considered safe while live ones can require further analyses of risk and benefit, especially for patients on immunosuppressive agents in whom usually live attenuated vaccines should be avoided. On the contrary, Varicella Zoster Virus (VZV) vaccination is required before initiating treatment with certain disease modifying treatments, such as glucocorticoids, methotrexate and biologics [29]. Moreover, as far as tetanus vaccine is concerned, there is evidence that it can reduce the probability of MS relapse [30].

### Apnea in pre-term newborns (APTN)

**How is a case of APTN defined and how frequent is it?**

An apneic episode is usually defined as a cessation of breathing for 20 seconds or longer or a shorter breathing pause accompanied by bradycardia (< 100 bpm), cyanosis or pallor [31].

It is a significant clinical problem manifested by an unstable respiratory rhythm reflecting the immaturity of respiratory control systems. Anatomically the immaturity is manifested as decreased synaptic connections, decreased dendritic arborization and poor myelination. The condition should be considered a developmental disorder rather than a disease state because it resolves with maturation [32]. All infants born at ≤ 28 weeks' gestation were diagnosed with apnea, while 85% of those born at 30 weeks and 20% of those born at 34 weeks were diagnosed with apnea [31].

Regarding post-immunization APTN, some authors estimate an incidence rate of post-immunization cardiorespiratory events (apnea, bradycardia and/or desaturation) between 7 and 47% [33-35], whereas for apnea alone the incidence varies between 10 and 20% [36, 37].

**What are the pathophysiological mechanisms underlying APTN?**

Pathophysiological hypotheses are uncertain and not related to a specific vaccine or to adjuvants. An increase in systemic inflammatory response after immunization interacting with an immature nervous system in the preterm newborn has been postulated. In support of this hypothesis studies have shown that there is a temporal correlation between immunization and an increase in interleukin 6 (IL-6) and C-reactive protein (CRP) [38-41].

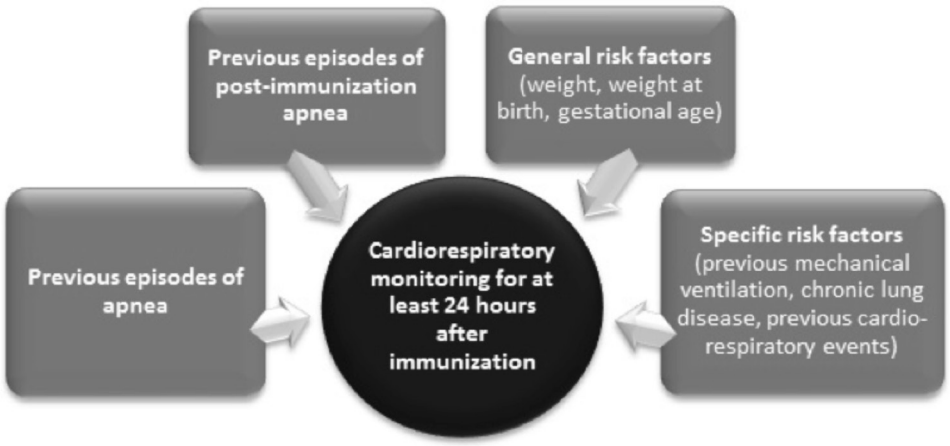
**Which are the vaccines associated with APTN onset?**

Vaccines associated with APTN are: diphtheria-tetanus-whole cell pertussis, *H. influenzae b*, Hepatitis B Virus, Inactivated Polio Virus, Meningococcal C conjugate (DTwP, DTwP-Hib, DTwP-Hib-HBV-IPV and MenC) [34-37, 42, 43]. As far as pertussis vaccine is concerned, it has recently been shown that the incidence of APTN post-diphtheria-tetanus-acellular pertussis (DTaP) immunization is similar to the post-DTwP incidence of APTN [44-46].

**What are the risk factors for APTN and how do they affect immunization?**

Several risk factors have been identified: age [35, 43, 44], weight [34, 37, 43], gestational age [44], previous mechanical ventilation [34, 35], presence of chronic pulmonary pathology [34], history of cardiorespiratory events [44, 46], pre-immunization apnea episodes [47]. Some studies have highlighted that the association between apnea and immu-

Fig. 2. Main factors affecting the need for cardiorespiratory monitoring after immunization.




nization is significant if immunization occurred before 67 days of age, whereas significance disappears for immunization after this time period [47, 48]. It is therefore advisable to perform cardiorespiratory monitoring after vaccination of all pre-term infants with risk factors [47].

**What is the recommended action regarding immunization in case of previous episodes of APTN?**

As with all preterm newborns, immunization is very important and should be performed according to chronological age. If possible the first vaccination should be performed before the discharge from the hospital [49-51].

It is advisable to perform cardiorespiratory monitoring after vaccination particularly for those who experienced pre-immunization apnea who are at 25-fold risk of developing post-immunization apnea compared to those who did not [47]. In order to avoid recurrent episodes in preterm infants who experienced post-immunization apnea, cardiorespiratory monitoring for at least 24 hours after vaccination is recommended [41] (Fig. 1). However, the episodes of post-vaccination apnea are self-limiting and do not cause long-term sequelae [44, 52] (Fig. 2).

## Guillain-Barré Syndrome (GBS)

**How is a case of GBS defined and how frequent is it?**

Guillain-Barré syndrome (GBS) is a rare condition in which a person's immune system attacks their peripheral nerves. The syndrome can affect the nerves that control muscle movement as well as those that transmit feelings of pain, temperature and touch, determining muscle weakness and loss of sensation in the legs and/or arms. It is more common in adults and in males; however, people of all ages can be affected. Around 3-5% of GBS patients die from complications like paralysis of the muscles of respiration, blood infection, lung embolus or cardiac arrest [53, 54]. Etiology of GBS is still unknown, but infections from influenza, Cytomegalovirus, Zika Virus and *C. jejuni* can act as triggers for the onset of GBS [55-59].

**What are the pathophysiological mechanisms underlying GBS?**

Mechanisms are uncertain, but thought to be immune mediated and based on auto-immune reactions: immunological similarity [60-62], production of anti-myelin or anti-axonal glycoproteins antibodies induced by vaccine epitopes [63]. Alternatively it is also hypothesized that vaccine components can interfere directly with peripheral nervous system structures [64]. The time frame of onset considered for possible vaccine-induced GBS is commonly up to 6 weeks after vaccination, but some authors consider a longer time-frame (Fig. 1).

**Which are the vaccines associated with GBS onset?**

Several studies investigated the correlation between influenza vaccine and GBS occurrence, with mixed results (Tab. II).

Over the last years cumulative evidence on the increased incidence of GBS following H1N1 influenza vaccination in the 2009-2010 season was reported with a significantly higher risk for non-adjuvanted *vs* adjuvanted vaccine [68, 70]. A European multinational case-control study on the same vaccine showed an increased risk of GBS post-vaccination, but after adjusting for upper respiratory tract infections, influenza-like illness or influ-

Tab. II. GBS incidence in different influenza seasons (vaccinated vs non vaccinated subjects).

| Influenza seasons | GBS incidence in vaccinated subjects (per million) |
|---|---|
| 1976 (H1N1) | 7.2 cases per million in the vaccinated group vs 0.79 cases per million in the non-vaccinated group [64] |
| 1991-1999 | 0.95 cases per million in the group vaccinated against influenza vs 0.22 cases per million in the group vaccinated against diphtheria-tetanus [65] |
| 1990-2005 | 0.7 cases per million vaccinated subjects [66] |
| 2000 | 0.86 cases per million vaccinated subjects [67] |
| 2001 | 1.21 cases per million vaccinated subjects [68, 69] |
| 2009 (H1N1) | 1.6 excess cases per million vaccinated subjects [68, 69] |

enza vaccination, the association was no longer significant [68, 71].
In this regard, epidemiological data from the available literature note that the relative risk (RR) of GBS following vaccination is far lower than the one following an infectious disease (RR 1.41 *vs* 7.35 [68, 72-75]), especially in the case of influenza [76].
According to a recent simulation, assuming a typical influenza season and a typical vaccine effectiveness, influenza vaccination would reduce the individual risk of GBS [77, 78] (Tab. II).
In 2011, the Institute Of Medicine (IOM) could not confirm nor refute the causal relationship between GBS and anti measles, mumps, rubella, varicella, Hepatitis A Virus, Hepatitis B Virus, Human Papilloma Virus (HPV), influenza, diphtheria, tetanus and acellular pertussis vaccines because of inadequacy of available data. In 2004 the IOM confirmed the causality between the 1976 influenza vaccine and GBS [8].

**What are the risk factors for GBS and how do they affect immunization?**

Concerning risk factors for post-vaccination GBS, the main one identified to date is the previous occurrence of GBS or the presence of GBS at the time of immunization [79, 80]. Therefore it is important to collect an adequate clinical history asking for previous occurrences of GBS.

**What is the recommended action regarding immunization in case of previous episodes of GBS?**

In individuals with a history of GBS, the risk of recurring GBS requiring hospitalization after vaccine administration is estimated at 1.18% in a study [81] and at 3.7% in another [80]. At the present time, the ability of influenza vaccination to cause GBS is not an established scientific finding. However, as a precautionary measure, it is advised not to administer vaccines to individuals who have experienced GBS in the previous 6 weeks unless they are at high risk for severe influenza complications; alternatively, the possibility of antiviral chemoprophylaxis can be considered in these subjects [79, 80]. For all other patients with a history of GBS outside of the 6 weeks interval, the decision to vaccinate should be based on risk-benefit balance assessment.

## Vasculitides

**How is a case of vasculitis defined and how frequent is it?**

Vasculitides are a group of related disorders characterized by inflammation of blood vessels leading to tissue or end-organ injury with diverse and only partially understood etiology and with a wide spectrum of clinical manifestations and prognoses [82].
Vasculitis in children is rare. The annual incidence is estimated at about 53/100,000 subjects [83]. The type of vasculitis depends primarily on age: in pediatric populations the most frequent vasculitides are Henoch-Schönlein purpura (annual incidence: 10-20/100,000) and Kawasaki disease (annual incidence: 1-19/100,000; around 1/100 in Japanese newborns); in adults the most common is hypersensitivity vasculitis. Regarding the epidemiology of the different forms of vasculitis, variations are found depending on ethnic and geographic factors: Microscopic Polyangiitis occurs more frequently in Asia, Wegener's granulomatosis in North America and northern Europe, Takayasu arteritis in Japan and Horton's arteritis in Europe and North America. Almost all types of vasculitides have been tentatively implicated as AEs but only in a few there is evidence supporting this association [84].

**What are the pathophysiological mechanisms underlying post-immunization vasculitides?**

A causality relationship has not been established with certainty; the main hypothesis rests on the concept of molecular mimicry associated with circulating immune-complex deposition [85].
Notwithstanding the uncertainty on the basic pathophysiological mechanism, in some cases of arthritis/arthralgia and vasculitides a mechanism can be found.
In 2011 the term Autoimmune/inflammatory Syndrome Induced by Adjuvants (ASIA) [86] was coined to describe a group of autoimmune-derived clinical conditions with similar symptoms, that occur after vaccination, supposedly induced by adjuvants [86-88]. Underlying the onset of ASIA, an individual predisposition [89] and an environmental triggering factor (endogenous or exogenous) are necessary conditions. Molecular mimicry and overstimulation of the immune system are the pathophysiological mechanisms thought to be responsible for ASIA occurrence [90].

**Which are the vaccines associated with vasculitides onset?**

Over the last years several articles have been published on the correlation between vaccinations and vasculitides. The most frequently considered immunizations were influenza, Hepatitis B Virus, Bacillus Calmette-Guérin, Human Papilloma Virus, Meningococcal C conjugate and Hepatitis A Virus vaccines, while potentially correlated vasculitides were mostly cutaneous vasculitides, Henoch-Schönlein Purpura (HSP), Kawasaki Disease (KD). Fewer studies were performed on Systemic Lupus Eritematosus (SLE), ANCA-Associated Vasculitides (AAV), Giant Cell Arteritis (GCA) and Polyarteritis Nodosa (PAN) [82].
At the present time the only associations that have not been rejected in evidence reviews are the following [84]:

- influenza vaccine and cutaneous vasculitides [91];
- influenza vaccine and GCA [92];
- Hepatitis B Virus (HBV) vaccine and Polyarteritis Nodosa (PAN) [85].

In particular, the association between vaccination and Kawasaki Disease, which is the second most frequent pediatric vasculitis [83] has been investigated in depth in the last few years. However, this possible association has been rejected based on assessments of Vaccine Adverse Events Reporting System (VAERS) data in 2009 [93] that demonstrated the absence of a temporal relationship between any vaccination and onset of KD symptoms. In spite of this evidence, the fear of an association of this disease with vaccination has remained high and pushed the Food and Drug Administration (FDA) to include it in the pentavalent anti Rotavirus vaccine adverse events re-

porting, following the occurrence of 5 cases out of over 36,000 vaccinated children during phase 3 research [94]. Another vaccination that has been associated with KD is Meningococcal B vaccine for which regulatory authorities have requested ad-hoc post-marketing safety studies [95].

Finally, in 2015 a multicentric study was unable to demonstrate an association between specific vaccinations and KD [96].

**What are the risk factors for post-immunization vasculitides and how do they affect immunization?**

Individual predisposition appears to be fundamental in the onset of these disorders [84].

A personal or family history of autoimmunity can represent a risk factor for certain vasculitides [97]. For precautionary reasons, it is advisable to follow subjects at risk for Giant Cell Arteritis (GCA) or Polymyalgia Rheumatica, depending on gender or age, for 2-6 months after influenza vaccination [92] (Fig. 1).

**What is the recommended action regarding immunization in case of previous or current episodes of vasculitides?**

There is mounting evidence that some infections can act as triggers for the onset of vasculitides, particularly Hepatitis B and C (HBV and HCV) viruses for PAN and respiratory tract infections for Henoch-Schönlein Purpura (HSP). Infections have also been implicated in the relapse of some vasculitides, so that influenza and pneumococcal vaccinations are recommended in affected subjects [98, 99], while in France a successful campaign of vaccination against HBV was followed by a decrease in the incidence of Polyarteritis Nodosa (PAN) [98].

### Arthritis/Arthralgia (AA)

**How is a case of AA defined and how frequent is it?**

The term arthralgia is used in the medical literature to indicate articular pain in general; in some cases arthralgia refers to a non-inflammatory condition of the joint, but this is not consistent in the literature. Among the causes of arthralgia are traumatic injury, degenerative processes of the joint, systemic inflammatory disorders, lupus, rheumatoid arthritis and vaccinations [100].

The word arthritis is used by clinicians to specifically mean inflammation of the joints, while it is used in public health to refer more generally to more than 100 rheumatic diseases and conditions that affect joints, the tissues that surround the joint, and other connective tissue, and typically characterized by pain and stiffness in or around one or more joints. The pattern, severity, location of symptoms, involvement of the immune system and other internal organs varies depending on the type of disease [101]. In 2012 in the US 52.5 million adults aged ≥ 18 years had self-reported or doctor-diagnosed arthritis, and 22.7 million reported arthritis-attributable activity limitation [102].

**What are the pathophysiological mechanisms underlying post-immunization AA?**

Several models have been proposed: a direct mechanism through polyclonal B cells activation and functional alteration of immune-regulatory cells and an indirect mechanism through the production of cytokines, molecular mimicry and immune-complex formation [103]. However, pathophysiological mechanisms post-immunization remain uncertain, as well as the period of onset (Fig. 1).

Possible mechanisms that have been postulated to cause in some cases the onset of post-immunization AA are the ones at the root of ASIA (See the section: "What are the pathophysiological mechanisms underlying post-immunization vasculitides?")

**Which are the vaccines associated with AA onset?**

Many vaccines, such as anti-Hepatitis A and B Viruses, measles, mumps, rubella, Varicella Zoster Virus and Human Papilloma Virus (HAV, HBV, MMR, VZV and HPV), have been implicated in the occurrence of AA as they can function as an exogenous trigger for the development of autoimmune disorders [100, 105]. However, the role of vaccinations as possible causative agents of AA has not been established with sufficient evidence. In spite of this, many isolated cases or series of cases of arthritis following vaccination have been reported. These cases tend to be very infrequent and usually only short-term outcomes are described [106]. In fact, the occurrence of post-vaccination arthritis/arthralgia (AA) is usually self-limiting and of moderate intensity [90, 104, 105].

**What are the risk factors for post-immunization AA and how do they affect immunization?**

In the case of rubella vaccination, possible risk factors are: advanced age, Human Leucocyte Antigens (HLA) dependent predisposition and female gender. Further studies however, found no evidence of increased risk for chronic arthropathy among women vaccinated against rubella.

However, in the absence of scientific evidence of the causal relationship between vaccination and AA and considering the overall risk-benefit balance, it can be stated that vaccination for the overwhelming majority of patients carries no risk of systemic autoimmune disease and should be administered according to current recommendations [105, 107, 108].

**What is the recommended action regarding immunization in case of previous episodes of AA?**

Previous occurrence of episodes of AA or the presence of AA at the time of immunization is an important risk factor for the onset of post-immunization AA. However, for individuals suffering from AA, vaccination is still recommended because natural infection can cause a relapse of the AA symptoms. In order to give more information on timing and vaccination type to be administered, several studies were conducted in recent years [109-115]:

- vaccination should be performed during the remission phase of the disorder and can be done in patients taking Disease Modifying Anti-Rheumatic Drugs (DMARDs) and anti-Tumor Necrosis Factor (TNF) drugs;
- vaccination should be performed before starting therapy with anti-B cells drugs;

- the administration of live attenuated vaccines, such as BCG, should be avoided, particularly in immunosuppressed individuals;
- some studies have shown efficacy and safety of non-live vaccines in subjects whose condition is under pharmacologic control, even in those taking biologic drugs; influenza and pneumococcal vaccines are strongly recommended in these patients and the choice of vaccine should be as personalized as possible since different Human Leucocyte Antigens (HLA) genetic polymorphisms cause inter-individual variation in terms of efficacy and toxicity.

It should be noted that, although vaccination is strongly recommended in these subjects, post-immunization relapse or symptoms exacerbations can occur, possibly through a mechanism of polyclonal activation of B-cells or through cross-reactivity [90].

## Immune Thrombocytopenic Purpura

**How is a case of Immune Thrombocytopenic Purpura (ITP) defined and how frequent is it?**

The term refers to an autoimmune disorder of unknown etiology characterized by a platelet count < 100,000/μl and by the presence of small areas of hemorrhage (purpura).

Data from surveillance system reports indicate that the frequency and severity of vaccine induced immune thrombocytopenic purpura (VI-ITP) is much lower than after natural infection from vaccine-preventable diseases [116-119].

The incidence of VI-ITP is probably underestimated because mild to moderate and asymptomatic cases often do not come to the attention of physicians and are therefore not diagnosed and reported [120, 121].

**What are the pathophysiological mechanisms underlying vaccine induced immune thrombocytopenic purpura (VI-ITP)?**

The occurrence of thrombocytopenia has been convincingly related to the production of antibodies that cross-react with platelet antigens that can be detected in about 80% of cases [117-122]. In the case of thrombocytopenia that occurs after anti measles-mumps-rubella (MMR) vaccination, the presence of anti-rubella and anti-measles IgG antibodies that cross-react with platelet antigens has been consistently detected [123-125]. For these reasons the pathophysiological mechanisms underlying VI-ITP can be considered certain.

**Which are the vaccines associated with VI-ITP onset?**

Specific studies have been performed to assess VI-ITP associated with measles-mumps-rubella (MMR) and Hepatitis B Virus (HBV) vaccination, since the occurrence of immune thrombocytopenic purpura (ITP) is a possible complication of measles and HBV infection. Regarding HBV vaccine, VI-ITP can occur after any dose of the vaccine and, in case of recurring episodes, the clinical picture tends to worsen, thus requiring specific treatment [126]. VI-ITP that occurs after MMR vaccination has an onset within 6 weeks of vaccine administration and presents with a higher platelet count than ITP after natural infection [127] and with clinical manifestations such as petechiae of moderate severity [128], although rarely there have been reports of gastrointestinal (GI) and/or pulmonary hemorrhage [129], hematuria [130] and the need for splenectomy [131] (Fig. 1). The forms of VI-ITP that require hospitalization have a lower average duration of hospitalization than those caused by natural infection (3 *vs* 5 days) [133-135] and no deaths strictly correlated with VI-ITP after MMR vaccination have been reported, unlike in the case of natural infection [117-121]. In the majority of cases (about 90%), VI-ITP is self-limiting within 6 months of diagnosis and only 10% turns into a chronic condition [127-128], whereas ITP after viral infection can become chronic in about 25% of cases on top of having a more severe clinical course [136]. A study performed on a cohort of 1.8 million children and adolescents (age range: 7 weeks to 17 years of age) investigated the correlation between all vaccinations and the onset of ITP, considering a time frame from the date of immunization to 6 weeks after immunization. The results indicate an increase in risk after Hepatitis A Virus and Varicella Zoster Virus vaccinations performed between the 7$^{th}$ and 17$^{th}$ year of age and the 11$^{th}$ and 17$^{th}$ respectively [137] (Tab. III).

**What are the risk factors for VI-ITP and how do they affect immunization?**

The main risk factor for the onset of an episode of VI-ITP is a previous episode of Immune Thrombocytopenic Purpura (ITP) or VI-ITP or the presence of ITP/VI-ITP at the time of immunization [117-121] (see below).

**Tab. III.** Increase of cases of Immune Thrombocytopenic Purpura (ITP) and their clinical course by selected vaccines.

| Vaccine | Age range during which an increase in cases after vaccination occurred | Clinical course |
|---|---|---|
| MMR [137] | 12th-19th month of age, 1 additional case every 40,000 doses (p = 0.006); no changes with MMVR use | Usually moderate severity, in exceptional cases GI and/or pulmonary hemorrhage. If hospitalization required, hospital stay shorter than in post-infection ITP |
| HAV [137] | 7th-17th year of age (p = 0.001) | * |
| VZV [137] | 11th-17th year of age (p = 0.04) | * |
| HBV [126] | Inadequate evidence | Lack of guidelines; episodes appear to recur and become more severe after each vaccine administration |

*Number of cases is insufficient to describe a typical clinical course.

**What is the recommended action regarding immunization in case of previous episodes of ITP or VI-ITP?**
In subjects with a history of ITP or post-measles-mumps-rubella (MMR) vaccine VI-ITP, with a normal platelet count at the time of immunization, MMR vaccine appears to be safe and well tolerated [117-121], although relapse of ITP can occasionally occur [138, 139]. Recent studies claim that the first MMR dose does not normally trigger a relapse of ITP and that the booster dose is not followed by relapse within 6 weeks of administration [117-121]. However, the assessment of antibody titer against measles, mumps and rubella is recommended in patients with chronic ITP or with previous post-MMR VI-ITP to avoid further vaccine doses if the antibody titer is protective [119, 140, 141].
International guidelines on immune thrombocytopenia management recommend vaccination against *S. pneumoniae*, *H. influenzae B* and *N. meningitides* before splenectomy [142].

## Commentary and conclusions

As shown in this review, the issue of adverse events in the field of immunization is subject to a lot of uncertainties, especially as regards rare and very rare ones; indeed, it is often not possible to conclude with sufficient scientific rigor on causal relationships (or the lack thereof) between vaccines and adverse events [8]. This poses a significant problem in risk communication and risk management as health care workers need to provide fair and balanced information to patients while at the same time not discouraging immunization. Unfortunately, the asymmetry between the knowledge about benefits and the knowledge about risks is common throughout medicine.
It is important to keep in mind that the adverse events we have covered occur rarely or very rarely but that, at the same time, the benefits are not directly experienced by the individual who is vaccinated and that the tolerance for risks (real or perceived) in our societies is relatively low [143].
With many additional vaccines being developed and introduced in vaccination schedules, and with the requirement to be immunized in order to be able to attend kindergarten and schools increasingly introduced or suggested to fight vaccine hesitancy [144], the emphasis on vaccine safety and adverse event monitoring becomes increasingly important. It is also crucial to correctly identify, as far as possible, those individuals who are at higher risk for adverse events and require the adoption of additional precautions and follow-up after immunization, and the few who need to be exempted for medical reasons. On the other hand, the current trend is against compulsory immunization (also on the basis of new evidence showing poor results obtained where both compulsory and voluntary vaccinations are simultaneously present in vaccine schedules) [145], while it is advised to counsel individuals in order to provide correct information on benefits and risks, thus empowering them to make an informed choice.
For these reasons, as some important scientific bodies have suggested, it would be vital to obtain greater knowledge on adverse events, possibly through the widespread use of electronic medical records that would enable a far higher sensitivity in epidemiologic assessments of rare/very rare adverse events. Collecting evidence that fulfills causality criteria [146] will help both risk communication and decision making.
Furthermore, the additional knowledge accumulated through "mechanistic" biomedical research can help elucidate in greater detail the pathophysiological mechanisms of vaccine induced harm and possibly help identify in advance the subjects at risk for harm, moving more and more towards tailored medicine.
Since there is no gold standard in risk communication in the field of vaccination, it is advisable to perform studies aimed at determining a more effective medium of communication [147]. It is important to bear in mind that vaccination risks should not be omitted both because it is unethical, and because considering a vaccine unsafe does not necessarily imply having doubts on its efficacy, therefore perceptions of vaccine importance may mitigate losses in vaccination uptake [148].
Trust in institutions such as Public Health and Government has effects on trust in immunization, and vice-versa [147, 148]; it is necessary to develop a new strategy of risk communication that, rather than showing lists of adverse events, disease complications and their incidence, makes use of widely intelligible instruments and language, in order to clearly answer the questions of both Health Care Workers and Public Health services users.

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AC, GG, LZ declare absence of conflicts of interest.

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ORIGINAL ARTICLE

# Epidemiology of herpes simplex virus type 1 and 2 in Italy: a seroprevalence study from 2000 to 2014

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Keywords

Herpes simplex virus • HSV-1 • HSV-2 • Seroprevalence • Italy

Summary

*Herpes simplex viruses (HSV) are among the most widespread causative agents of human viral infections. HSV-2 is one of the commonest causes of genital disease, while HSV-1 is associated primarily with orolabial ulceration; however, recent changes in HSV epidemiology showed an increase in genital and neonatal herpes particularly caused by HSV-1. The main purpose of this study was to assess the seroprevalence of HSV-1 and HSV-2 in a random population in Siena (central Italy) in 2000, 2005 and 2013-2014 and in Bari (southern Italy) in 2005. Moreover, a preliminary study was conducted to investigate the spread of HSV infection in a population of pregnant women and infants in Bari in 2003, 2004 and 2005. Human serum samples were tested for the presence of specific anti-HSV-1 and anti-HSV-2 IgG antibodies using a commercially available ELISA test. For the primary purpose, seroprevalence rates observed in Siena were compared over the years sampled and with the seroprevalence rate found in Bari. Results of seroprevalence in Siena show a decreased trend for both viruses, especially in adolescents and young adults; moreover, HSV-2 seroprevalence rates found in the two cities suggest geographical differences. For the secondary purpose, prevalence rates among pregnant women were compared with the seroprevalence found in women of the general population. No significant difference in prevalence rates were found among pregnant women, while results indicate both viruses are a source of infection in infants.*

## Introduction

Herpes simplex virus (HSV) is one of the most widespread infections in humans, affecting 60-95% of the adult population worldwide. After entering the host, the virus establishes persistent and latent infection in neuronal ganglia, from which it can reactivate periodically causing recurrent infections. Most infections are subclinical; symptoms consist primarily of ulcerative lesions at the site of infection and, although rare, complications such as blindness, encephalitis and aseptic meningitis can occur, especially in immunocompromised hosts [1, 2]. Moreover, the acquisition of HSV during pregnancy is associated with miscarriage, prematurity, and congenital and neonatal herpes. HSV can cause severe infections in newborn by vertical transmission in utero or, in most cases, during vaginal delivery through contact with HSV-infected genital tract secretions, with a high mortality and neurodevelopmental disability [3-5].

There are two types of HSV, HSV-1 and HSV-2, both transmitted by direct contact with infected secretions. HSV-2 is transmitted sexually, resulting in one of the most common genital diseases, affecting adolescents and adults and facilitating HIV transmission [6, 7]. HSV-1 infection is associated predominantly with orolabial ulceration and occurs mostly by nonsexual contacts during childhood after the disappearance of maternal antibodies in the first year of life [7, 8]. However, recent changes in herpes infection epidemiology reported an increase in genital and neonatal herpes due to HSV-1 [9].

Nevertheless, many infections remain asymptomatic or do not require health care intervention, resulting in an underestimation of the spread of HSV. Serological tests based on the use of type-specific immunoassays that distinguish antibodies to HSV-1 and HSV-2, can recognize both symptomatic and asymptomatic infections, facilitating a better understanding of HSV epidemiology [6].

In Italy, some studies have investigated the seroprevalence of these viruses in different populations considered to be at low or high risk of acquiring the infection. According to these studies, HSV-1 infection is widespread, with seroprevalence rates of 93% in the adult population [10]. Conversely, the circulation of HSV-2 is more limited (5.5% in adults, according to Suligoi et al. [11]) and frequently associated with other sexually transmitted diseases (STD) [12-14]. Moreover, a HSV-2 seroprevalence study conducted on samples collected in 1998 showed 7.6% positivity to HSV-2 antibodies among pregnant women [11], comparable to 8.4% seroprevalence in a similar survey conducted in Northern Italy [15].

HSV infection control is of relevance for public health especially for mitigating the risk of neonatal herpes and as-

sociated diseases; thus, monitoring the epidemiology of HSV infections is an important tool for prevention and control strategies, as suggested by Woestenberg et al. [16]. In Italy, a characterization of HSV infections in the general population over time is lacking. To revise the epidemiology of HSV in Italy, a seroepidemiological study was carried out to determine the HSV-1 and HSV-2 antibody prevalence in the general population of two cities from different geographical areas (Siena, central Italy and Bari, southern Italy), making a comparison between seroprevalence rates obtained over a period of nearly 15 years. Moreover, a preliminary study was carried out in a small population of samples of pregnant women and infants, to evaluate HSV as a source of infection in infants.

## Materials and methods

HSV antibody tests were performed on human serum samples from the internal serum bank of the Laboratory of Molecular Epidemiology, Department of Molecular and Developmental Medicine, University of Siena. The samples had been anonymously collected in compliance with Italian law on Ethics; the only information available for these subjects were age, gender and state of pregnancy.

1,776 samples (640 in 2000, 636 in 2005 and 500 in 2013-2014) collected in the Siena area and 168 samples collected in 2005 in the province of Bari were tested for the presence of specific anti-HSV-1 and anti-HSV-2 IgG antibodies. In addition, 91 samples from pregnant women and 70 samples from infants collected in the province of Bari in 2003, 2004 and 2005, were tested. In particular, infants were subdivided in two age-groups, 0-11 months of age and 1 year of age, considering the waning of maternal antibodies over a period of 6-12 months [17]. A population summary is shown in Table Ia, Ib and Ic.

Type specific serum antibodies to HSV-1 and HSV-2 were detected by commercial ELISA kits BEIA HSV 1 IgG and BEIA HSV 2 Rec IgG (TechnoGenetics, Milano, Italy). ELISA assays were performed in accordance with the manufacturer's instructions. According to the manufacturer, the tests have sensitivity and specificity of 98.7% and 100% for HSV-1 and 94.1% and 98.5% for HSV-2 respectively. Cut-off levels were > 1.1 for seropositivity and < 0.9 for seronegativity; samples with borderline results (between 0.9 and 1.1) were excluded from the study.

Sex and age-specific seroprevalence rates were calculated, along with the corresponding 95% CI. Statistical analysis was performed using the Yates corrected chi-squared test to compare prevalence rates among different groups and the chi-squared test for the trend to evaluate possible tendencies in seroprevalence rates over time. Statistical significance was set at $p < 0.05$, two tailed.

**Tab. I.** General population of Siena in 2000, 2005 and 2013-2014 (a), general population of Bari 2005 and pregnant women of Bari 2003, 2004 and 2005 (b) and infants of Bari 2003, 2004, 2005 (c).

a)

| Age groups | Siena 2000 | | | Siena 2005 | | | Siena 2013-2014 | | |
|---|---|---|---|---|---|---|---|---|---|
| | M | F | TOT | M | F | TOT | M | F | TOT |
| 0-14 | 60 | 40 | 100 | 39 | 19 | 58 | 10 | 5 | 15 |
| 15-19 | 8 | 41 | 49 | 10 | 10 | 20 | 9 | 13 | 22 |
| 20-24 | 12 | 39 | 51 | 10 | 11 | 21 | 23 | 22 | 45 |
| 25-29 | 12 | 42 | 54 | 7 | 29 | 36 | 26 | 23 | 49 |
| 30-34 | 16 | 50 | 66 | 11 | 28 | 39 | 19 | 31 | 50 |
| 35-39 | 20 | 31 | 51 | 16 | 37 | 53 | 25 | 36 | 61 |
| 40-49 | 43 | 63 | 106 | 39 | 59 | 98 | 51 | 59 | 110 |
| 50+ | 54 | 90 | 144 | 149 | 141 | 290 | 74 | 71 | 145 |
| Total | 225 | 396 | 621 | 281 | 334 | 615 | 237 | 260 | 497 |

b)

| Age groups | Bari 2005 | | | Bari 2003-2004-2005 |
|---|---|---|---|---|
| | M | F | TOT | Pregnant |
| 0-14 | 28 | 28 | 56 | - |
| 15-19 | 10 | 6 | 16 | - |
| 20-24 | 5 | 7 | 12 | 9 |
| 25-29 | 6 | 15 | 21 | 30 |
| 30-34 | 7 | 25 | 32 | 34 |
| 35-39 | 13 | 18 | 31 | 18 |
| Total | 69 | 99 | 168 | 91 |

c)

| Age groups | Bari 2003-2004-2005 |
|---|---|
| 0-11 months | 34 |
| 1 year old | 34 |
| Total | 68 |

## Results

A total of 2105 serum samples were tested by ELISA assay, 45 of which yielded borderline results and were excluded from the statistical analysis. Samples collected in the general population of Siena and Bari were divided by sex and classified into eight age groups: 0-14 (children), 15-19 (adolescents), 20-24 (young adults), 25-29, 30-34, 35-39, 40-49 and > 50 years old. Samples collected from pregnant women were divided in four age groups: 20-24, 25-29, 30-34 and 35-39 years old, while samples from infants were divided into 0-11 months and 1-year-old.

### HSV-1 seroprevalence in the general population

The results for specific IgG antibodies against HSV-1 in samples collected in Siena are reported in Table II.

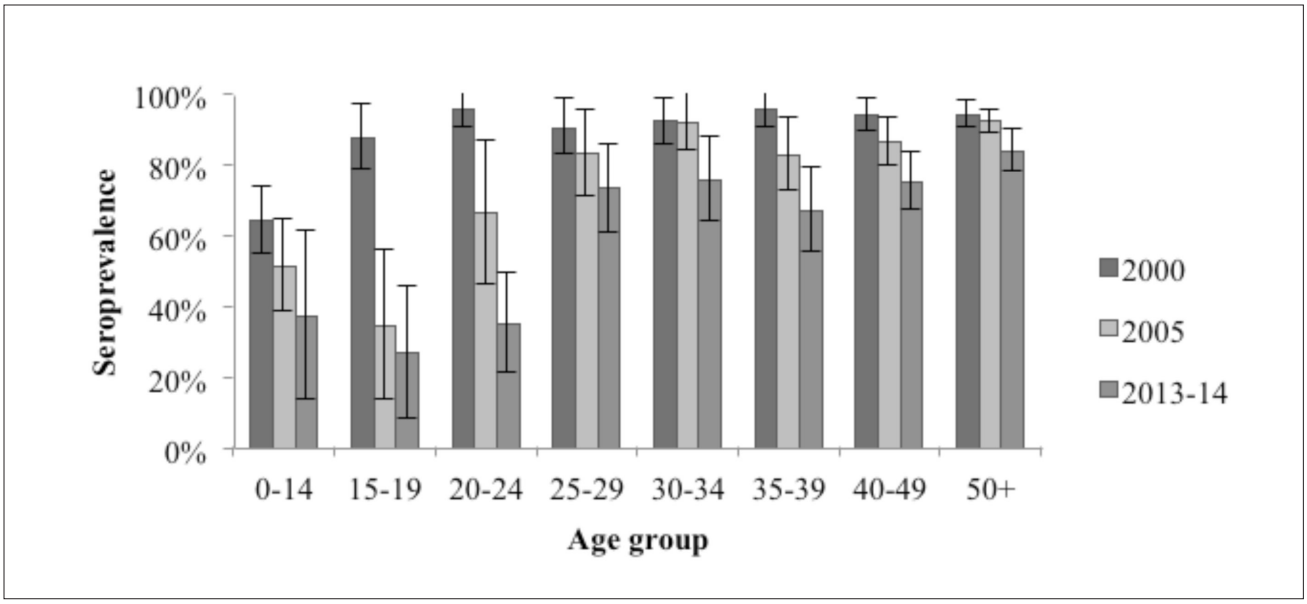
Overall, HSV-1 seroprevalence declined significantly ($p < 0.001$) from 2000 to 2013-2014 in the study population, in particular rates by sex showed a decrease of 14.5% in women and 22% in men; however, the prevalence remained significantly higher among women ($p = 0.003$).

In all the years considered in this study, a steady increase in the proportion of HSV-1 seropositive occurred

**Tab. II.** HSV-1 seroprevalence in population of Siena in 2000, 2005 and 2013-2014, divided by sex (male, M and female, F) and age groups.

| Age groups | Siena 2000 (%) | | | Siena 2005 (%) | | | Siena 2013-2014 (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | M | F | TOT | M | F | TOT | M | F | TOT |
| 0-14 | 42/60 (70) | 23/40 (57.5) | 65/100 (65) | 21/39 (53.8) | 9/19 (47.4) | 30/58 (51.7) | 3/10 (30) | 3/5 (60) | 6/15 (40) |
| 15-19 | 8/8 (100) | 35/41 (85.4) | 43/49 (87.8) | 6/10 (60) | 1/10 (10) | 7/20 (35) | 2/9 (22.2) | 4/13 (30.8) | 6/22 (27.3) |
| 20-24 | 10/12 (83.3) | 39/39 (100) | 49/51 (96.1) | 6/10 (60) | 8/11 (72.7) | 14/21 (66.7) | 7/23 (30.4) | 9/22 (40.9) | 16/45 (35.6) |
| 25-29 | 11/12 (91.7) | 38/42 (90.5) | 49/54 (90.7) | 5/7 (71.4) | 25/29 (86.2) | 30/36 (83.3) | 18/26 (69.2) | 18/23 (78.3) | 36/49 (73.5) |
| 30-34 | 14/16 (87.5) | 47/50 ( 94) | 61/66 (92.4) | 9/11 (81.8) | 27/28 (96.4) | 36/39 (92.3) | 10/19 (52.6) | 28/31 (90.3) | 38/50 (76) |
| 35-39 | 20/20 (100) | 29/31 (93.5) | 49/51 (96.1) | 15/16 (93.7) | 29/37 (78.4) | 44/53 (83) | 14/25 (56) | 27/36 (75) | 41/61 (67.2) |
| 40-49 | 38/43 (88.4) | 62/63 (98.4) | 100/106 (94.3) | 31/39 (79.5) | 54/59 (91.5) | 85/98 (86.7) | 36/51 (70.6) | 47/59 (79.7) | 83/110 (75.4) |
| 50+ | 47/54 (87) | 89/90 (98.9) | 136/144 (94.4) | 138/149 (92.6) | 131/141 (92.9) | 269/290 (92.8) | 58/74 (78.4) | 64/71 (90.1) | 122/145 (84.1) |
| Total | 190/225 (84.4) | 362/396 (91.4) | 552/621 (88.9) | 231/281 (82.2) | 284/334 (85) | 515/615 (83.7) | 148/237 (62.4) | 200/260 (76.9) | 348/497 (70) |

**Fig. 1.** HSV-1 seroprevalence in population of Siena in 2000, 2005 and 2013-2014, divided by age groups with 95%CI.




with age ($p < 0.001$). As shown in Figure 1, from 2000 to 2013-2014 seroprevalence declined significantly ($p < 0.001$) among adolescents and young adults (from 87.8% and 96.1% in 2000 to 27.3% and 35.6% in 2013-2014 respectively) as well as for 35-39- and 40-49-years-old groups; moreover, there is an important decrease among 30-34 years old ($p = 0.018$) and in subjects over 50 ($p = 0.003$). Among children and 25-29 years old subjects, there were no significant changes.

The greatest increase in infections in 2000 was amongst adolescents ($p = 0.006$ with children), whilst in 2013-2014 it was in the 25-29 years group (73.5%; $p < 0.001$ with 20-24 age group of the same year). In particular, a significant difference was found between 20-24 and 25-29 year old men ($p = 0.015$), but also in women ($p = 0.024$).

The seroprevalence in samples collected in Siena in 2005 was compared with the results of the samples collected in Bari in the same year (Tab. III). No significant differences were found between the two cities.

### HSV-2 seroprevalence in the general population

The results for specific IgG antibodies against HSV-2 in the samples collected in Siena are reported in Table IV.

HSV-2 seroprevalence was halved between 2000 and 2013-2014 (from 22.4% to 11.5%, $p < 0.001$). In 2000 the prevalence among males and females exhibited no difference; in almost 15 years, both decreased ($p < 0.001$), but in 2013-2014 the prevalence was higher among males than females, even if this difference is not considered to be statistically significant.

In almost all age groups, seroprevalence decreased in the time span considered. The greatest decreases were observed particularly in adolescents ($p = 0.005$) and young adults ($p < 0.001$); a decrease was observed also in children from a value of 8% in 2000 to 0% in 2013-2014, but the difference was considered to be not statistically significant. In contrast, in 35-39 and 40-49 year old subjects no significant differences over the years were observed.

Considering the period 2013-2014, HSV-2 seropositivity is distributed only among the general population over 20 years of age, but over the years it was possible to observe some differences in seroprevalence patterns (Fig. 2). While in 2000 30.6% of adolescents showed HSV-2 positivity and the peak of infection was achieved in 20-24 year old subjects (47.1%) with a decrease up to 13.7% in 35-39-year old group, in 2013-2014 none of the adolescents in the study showed positivity to the virus. In the same years, the 20-24 year old age group remains the most affected in conjunction with that of 25-29 year olds; also there is a second increase in seroprevalence in people over 35 years.

The seroprevalence in samples collected in Siena in 2005 was compared with the results of samples collected in Bari in the same year (Tab. V).

The seroprevalence observed in the two cities showed a significant difference in the general population (13.2%

**Tab. III.** HSV-1 seroprevalence in Siena and in Bari in 2005, divided by sex and age groups.

| Age groups | Siena 2005 (%) | | | Bari 2005 (%) | | |
|---|---|---|---|---|---|---|
| | M | F | TOT | M | F | TOT |
| 0-14 | 21/39 (53.8) | 9/19 (47.4) | 30/58 (51.7) | 18/28 (64.3) | 13/28 (46.4) | 31/56 (55.4) |
| 15-19 | 6/10 (60) | 1/10 (10) | 7/20 (35) | 5/10 (50) | 4/6 (66.7) | 9/16 (56.2) |
| 20-24 | 6/10 (60) | 8/11 (72.7) | 14/21 (66.7) | 4/5 (80) | 5/7 (71.4) | 9/12 (75) |
| 25-29 | 5/7 (71.4) | 25/29 (86.2) | 30/36 (83.3) | 3/6 (50) | 13/15 (86.7) | 16/21 (76.2) |
| 30-34 | 9/11 (81.8) | 27/28 (96.4) | 36/39 (92.3) | 6/7 (85.7) | 22/25 (88) | 28/32 (87.5) |
| 35-39 | 15/16 (93.7) | 29/37 (78.4) | 44/53 (83) | 12/13 (92.3) | 16/18 (88.9) | 28/31 (90.3) |
| Total | 62/93 (66.7) | 99/134 (73.9) | 161/227 (70.9) | 48/69 (69.6) | 73/99 (73.7) | 121/168 (72) |

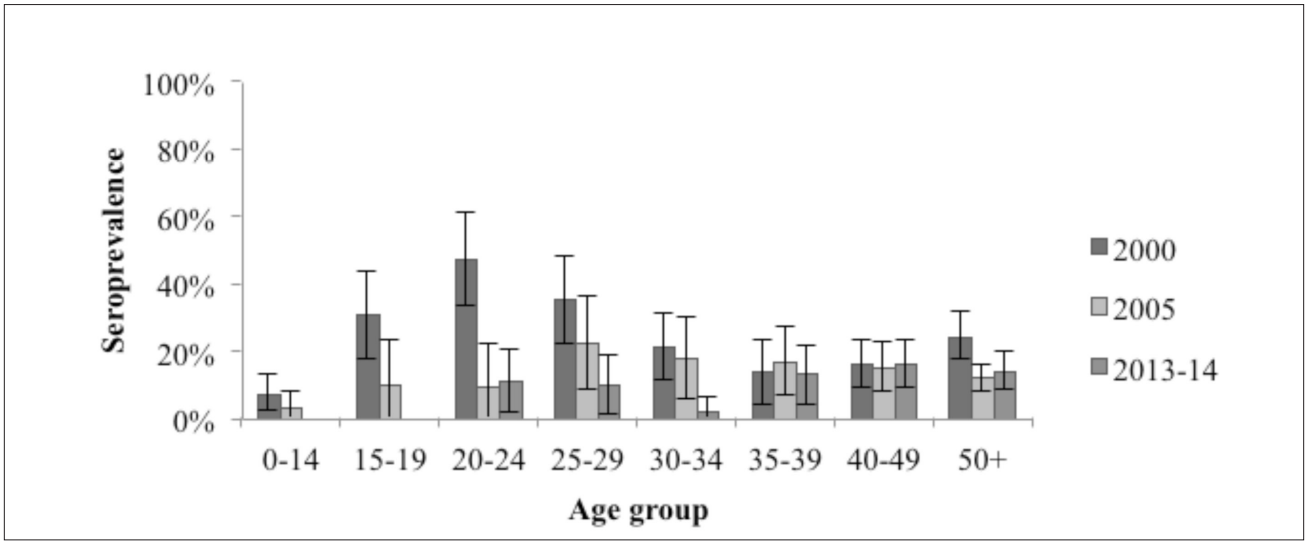
**Tab. V.** HSV-2 seroprevalence in Siena and in Bari in 2005, divided by sex and age groups.

| Age groups | Siena 2005 (%) | | | Bari 2005 (%) | | |
|---|---|---|---|---|---|---|
| | M | F | TOT | M | F | TOT |
| 0-14 | 1/39 (2.6) | 1/19 (5.3) | 2/58 (3.4) | 1/28 (3.6) | 1/28 (3.6) | 2/56 (3.6) |
| 15-19 | 1/10 (10) | 1/10 (10) | 2/20 (10) | 0/10 (-) | 0/6 (-) | 0/16 () |
| 20-24 | 1/10 (10) | 1/11 (9.1) | 2/21 (9.5) | 1/5 (20) | 0/7 (-) | 1/12 (8.3) |
| 25-29 | 1/7 (14.3) | 7/29 (24.1) | 8/36 (22.2) | 0/6 (-) | 2/15 (13.3) | 2/21 (9.5) |
| 30-34 | 3/11 (27.3) | 4/28 (14.3) | 7/39 (17.9) | 0/7 (-) | 2/25 (8) | 2/32 (6.2) |
| 35-39 | 2/16 (12.5) | 7/37 (18.9) | 9/53 (17) | 1/13 (7.7) | 0/18 (-) | 1/31 (3.2) |
| Total | 9/93 (9.7) | 21/134 (15.7) | 30/227 (13.2) | 3/69 (4.3) | 5/99 (5) | 8/168 (4.8) |

**Tab. IV.** HSV-2 seroprevalence in population of Siena in 2000, 2005 and 2013-2014, divided by sex and age groups.

| Age groups | Siena 2000 (%) | | | Siena 2005 (%) | | | Siena 2013-2014 (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | M | F | TOT | M | F | TOT | M | F | TOT |
| 0-14 | 5/60 (8.3) | 3/40 (7.5) | 8/100 (8) | 1/39 (2.6) | 1/19 (5.3) | 2/58 (3.4) | 0/10 (-) | 0/5 (-) | 0/15 (-) |
| 15-19 | 1/8 (12.5) | 14/41 (34.1) | 15/49 (30.6) | 1/10 (10) | 1/10 (10) | 2/20 (10) | 0/9 (-) | 0/13 (-) | 0/22 (-) |
| 20-24 | 7/12 (58.3) | 17/39 (43.6) | 24/51 (47.1) | 1/10 (10) | 1/11 (9.1) | 2/21 (9.5) | 3/23 (13) | 2/22 (9.1) | 5/45 (11.1) |
| 25-29 | 3/12 (25) | 16/42 (38.1) | 19/54 (35.2) | 1/7 (14.3) | 7/29 (24.1) | 8/36 (22.2) | 4/26 (15.4) | 1/23 (4.3) | 5/49 (10.2) |
| 30-34 | 6/16 (37.5) | 8/50 (16) | 14/66 (21.2) | 3/11 (27.3) | 4/28 (14.3) | 7/39 (17.9) | 1/19 (5.3) | 0/31 (-) | 1/50 (2) |
| 35-39 | 7/20 (35) | 0/31 (-) | 7/51 (13.7) | 2/16 (12.5) | 7/37 (18.9) | 9/53 (17) | 5/25 (20) | 3/36 (8.3) | 8/61 (13.1) |
| 40-49 | 4/43 (9.3) | 13/63 (20.6) | 17/106 (16) | 1/39 (2.6) | 14/59 (23.7) | 15/98 (15.3) | 9/51 (17.6) | 9/59 (15.2) | 18/110 (16.4) |
| 50+ | 16/54 (29.6) | 19/90 (21.1) | 35/144 (24.3) | 15/149 (10.1) | 20/141 (14.2) | 35/290 (12.1) | 11/74 (14.9) | 9/71 (12.7) | 20/145 (13.8) |
| Total | 49/225 (21.8) | 90/396 (22.7) | 139/621 (22.4) | 25/281 (8.9) | 55/334 (16.5) | 80/615 (13) | 33/237 (13.9) | 24/260 (9.2) | 57/497 (11.5) |

Fig. 2. HSV-2 seroprevalence in population of Siena in 2000, 2005 and 2013-2014, divided by age groups with 95% CI.




in Siena vs 4.8% in Bari, p = 0.008), in particular in women (p = 0.02).

### HSV-1 and HSV-2 seroprevalence among pregnant and infants

The prevalence of IgG antibodies against HSV-1 and HSV-2 in samples collected from pregnant women in Bari in 2003, 2004 and 2005 is shown in Table VI.

Among pregnant women, 91.2% and 9.9% showed antibodies against HSV-1 and HSV-2 respectively, while 8.8% were negative for both viruses. As shown in Table V, no significant differences were observed comparing pregnant and women of the same age of the general population collected in Bari 2005. In both populations, all subjects positive for anti HSV-2 IgG were also positive for anti HSV-1 IgG.++

Among infants, 38.2% and 30.9% showed anti-HSV-1 and anti-HSV-2 antibodies respectively (Tab. VII). 16.2% of them had antibodies directed against both viruses, in particular 23.5% of the infants 0-11 months of age and 8.8% of infants 1-year-old. HSV-1 seroprevalence showed a significant difference (p = 0.024) between infants 0-11 months of age and infants 1-year-old, while no difference was observed for HSV-2, or for co-detection of both viruses.

Tab. VI. HSV-1 and HSV-2 seroprevalence in Bari in 2003, 2004 and 2005 in pregnant women and not pregnant women, divided by age groups..

| Age groups | Pregnant | | No pregnant | |
|---|---|---|---|---|
| | HSV-1 (%) | HSV-2 (%) | HSV-1 (%) | HSV-2 (%) |
| 20-24 | 8/9 (88.9) | 2/9 (22.2) | 5/7 (71.4) | 0/7 (-) |
| 25-29 | 27/30 (90) | 5/30 (16.7) | 13/15 (86.7) | 2/15 (13.3) |
| 30-34 | 32/34 (94.1) | 1/34 (2.9) | 22/25 (88) | 2/25 (8) |
| 35-39 | 16/18 (88.9) | 1/18 (5.6) | 16/18 (88.9) | 0/18 (-) |
| Total | 83/91 (91.2) | 9/91 (9.9) | 56/65 (86.1) | 4/65 (6.1) |

Tab. VII. HSV-1 and HSV-2 seroprevalence in Bari in 2003, 2004 and 2005 among infants 0-11 months and 1-year-old.

| Age groups | HSV-1 (%) | HSV-2 (%) |
|---|---|---|
| 0-11 months | 18/34 (52.9) | 11/34 (32.3) |
| 1 year old | 8/34 (23.5) | 10/34 (29.4) |
| Total | 26/68 (38.2) | 21/68 (30.9) |

## Discussion

HSV-1 and HSV-2 are among the most widespread human viral infections. HSV-2 is one of the most common causes of genital disease, while HSV-1 is associated primarily with orolabial ulceration; however, recently an increase in genital and neonatal herpes caused by HSV-1 has been reported [7-9]. In Italy, the seroepidemiology of HSV infection has been investigated in populations associated with some risk factors, such as HIV infection [13] or other STDs [12, 14, 18], or considering a single serotype [10, 11].

The serological study presented here was conducted in order to estimate the seroprevalence of HSV-1 and HSV-2 in a general population from Siena, analyzing potential differences in the spread of infection from 2000 to 2014 and making a comparison with the seroprevalence in a general population from Bari. Moreover, a preliminary study was carried out in a small population of samples of pregnant women and infants, to investigate the spread of HSV as infection source of infection in infants.

As expected, analysis of data obtained from this study confirms the widespread prevalence of HSV-1 in the general population with seroprevalence rates higher than those to HSV-2.

The seroprevalence of HSV-1 declined significantly in the general population of Siena from 88.9% in 2000 to

70% in 2013-2014. This decrease has also been observed in other industrialized countries [6, 7, 16, 19] and associated with changes in socioeconomic status and family size, with improvements in living and hygiene conditions, as suggested by the same studies. The strongest decrease is observed in adolescents and young adults; in 2000 the peak of infection was achieved among adolescents, while in 2013-2014 HSV-1 infection was mainly acquired in 25-29 year-old subjects. Lack of HSV-1 immunity at the start of sexual activity makes young people more susceptible to genitally acquired HSV-1 infection [6, 19, 20]; women in particular are more susceptible to acquiring HSV-1, as well as HSV-2, as a genital infection because of a more vulnerable mucosal lining of the external genitalia [21]. In this study it was not possible to distinguish orolabial infections from genital infections. However, considering the 2013-2014 population, in every age group women show a higher rate of positivity than males of the same age, especially in the 30-34-year-old group.

HSV-2 prevalence declined significantly from 22.4% in 2000 to 11.5% in 2013-2014 among the general population and, as with HSV-1, the strongest decrease is observed in adolescents and young adults. In samples collected in 2000, 30.6% of adolescents exhibited positivity to HSV-2 antibodies, and the peak of infection was reached among young adults, while in 2013-2014 subjects who showed positivity to HSV-2 antibodies were over 20 years old. These results may reflect changes in sexual behavior, as acquisition of HSV-2 is considered a marker of previous sexual activity [6]. A second increase in HSV-2 seroprevalence was observed in people over 35 years of age without significant differences over the years, potentially correlated to a cumulative "sexual exposure" in older age groups. Considering the 2013-2014 population, women have a lower seroprevalence value than men, even if the difference is not statistically significant. This figure is in contrast with what has been observed by Pebody et al. [6], which states that women generally have a higher seroprevalence than men. A possible explanation could be that prior HSV-1 infection may protect against HSV-2 by conferring cross-immunity [7, 8, 22], even if it is still not known if a previous HSV-1 oral infection could confer the same protection as a HSV-1 genital infection.

While no differences were found among the general population of Siena and Bari for HSV-1 seroprevalence, HSV-2 antibody prevalence in Siena was higher than in Bari, especially in women, and additionally higher than those reported in Rome by Suligoi et al. [11]. These geographic differences could reflect historical differences in sexual behavior, as suggested by Pebody et al. [6], or differences in prevention and control programmes designed into these population.

Among pregnant women 91.2% and 9.9% showed antibodies against HSV-1 and HSV-2 respectively. HSV-2 seroprevalence in this study is consistent with those observed in Rome (7.6%) and Northern Italy (8.4%) [11, 15], but nevertheless it is lower than that reported among pregnant women in other countries, such as Germany (18%) and Finland (15.7%). Conversely, HSV-1 seroprevalence was higher (82% in Germany and 70% in Finland) [7, 23]. 8.8% of the pregnant women in this study are negative to both viruses and therefore are at greater risk to acquire the infection during pregnancy and transmit it to a newborn [5, 24], considering that approximately 3% of women in Italy acquires HSV infection during pregnancy [25].

These data are confirmed by seroprevalence rates found in infants: 52.9% and 32.3% of children of 0-11 months of age show antibodies against HSV-1 and HSV-2 respectively, resulting from an infection or inherited passively from the mother. After the first year of life HSV-1 seroprevalence declines to 23.5%, while 29.4% of infants show antibodies against HSV-2. Because maternal antibodies wane within the first year of life [17], the presence of anti HSV-1 or HSV-2 antibodies in children 1-year-old is indicative of infection. Comparing seroprevalence rates among infants 0-11 months and infants 1-year-old it could be postulated that the presence of anti-HSV-1 antibodies in the first months of life could be associated with infection or passively acquired maternal immunity, whilst antibodies directed against HSV-2 are almost always associated with HSV-2 infection, which is vertically transmitted more frequently than HSV-1 as suggested by other studies [5, 6].

The main importance of this study is that the detection of specific antibodies against HSV-1 and HSV-2 was conducted on samples that had been randomly collected in a general population, allowing characterization of the spread of the infections not only in high-risk subgroups (e.g. STD clinic attendees). On the other hand, some age groups are numerically limited because of the method of the sample collection and the lack of information (e.g. marital status, living area, education level, employment, sexual activity and number of partners, symptoms or previous diagnosis of genital herpes) on the subjects involved did not allow an evaluation of the influence of factors that may be related to an increased risk of HSV infection. Unfortunately, no data about the circulation of HSV-1 and HSV-2 in the years between those studied were available, therefore it was not possible to assess any changes in trends or possible epidemic outbreaks in the population studied.

## Conclusions

In conclusion, this study highlights a decreased trend in seropositivity for both HSV viruses, especially in adolescents and young adults, updating data from previous studies and providing important information about the changing epidemiology of these infections in Italy as already observed in other countries. Moreover, prevalence rates found among pregnant women and infants indicate both viruses as a source of infection in infants, emphasizing the importance of prevention with vaccine development and adequate communication to healthcare workers and public.

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## Authors' contributions

SM and CMT performed laboratory work. SM wrote the first draft of the manuscript. NT performed the linguistic revision of the manuscript. All authors critically read and revised the manuscript and approved the final version.

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ORIGINAL ARTICLE

# Microbial competition in environmental nosocomial reservoirs and diffusion capacity of OXA48-*Klebsiella pneumoniae*: potential impact on patients and possible control methods

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Keywords

Control; hospital-reservoir • OXA48-*K. pneumoniae* • VIM-*Enterobacteriaceae*

Summary

**Background**. *We have found clusters of Klebsiella pneumoniae with OXA48-carbepenemase cases in some hospital rooms, and decided to investigate whether bathroom siphons could be a reservoir for OXA48 bacteria, as occurs with K. oxytoca with other types of carbepenemases.*
**Methods**. *We evaluated the microbial competition between strains with OXA48 and VIM carbepenemases, in diluted nutrient-broth, on a slime germ-carrier. We compared the number of colonies at 5 and 10 days on the contaminated carriers with one or two strains. We evaluated the dissemination of K. pneumoniae with carbepenemase OXA48 or VIM from thumbs and index fingers of volunteers, to standard surfaces (20 glass germ-carrier by each volunteer). After, we counted the number of microorganisms on each carrier. Microbiological weekly studies of faecal microbiota of all patients were obtained in Traumatology and Oncology. Moreover, we studied samples of the sink in their rooms. PCR and MLST sequence-type was determined in all K. pneumoniae diagnosed from patients and sinks.*
**Results**. *A large possibility of diffusion from contaminated hands, which continue to transmit high numbers of microorganisms after more than 10 successive surface contacts, was highlighted; OXA bacteria were more persistent than VIM bacteria. Microbial competition studies showed that VIM bacteria are inhibited by OXA ones. These observations can explain the concentration of cases of K. pneumoniae OXA48 in some rooms in Traumatology and Oncology, producing a significant OR between rooms with OXA48-bacteria-contaminated siphons and other rooms (3.1 and 3.3 respectively). Risk was lowered after changing or disinfecting (heat plus chlorinated disinfectant) the contaminated siphons. Siphon colonization by VIM bacteria was not related with human infections by similar microorganisms.*
**Conclusions**. *Bathroom siphons can be a reservoir for K. pneumoniae OXA48 and lead to outbreaks. Outbreaks can be controlled by replacement or heat plus chemical treatment of the sink-siphons.*

## Introduction

Environmental contamination in hospitals has become increasingly important given our patients' higher susceptibility to infection because of several concurrent causes such as increasing age, multiple pathologies, longer hospital stays, more and longer instrumentation periods or treatments with broad spectrum antibiotics. These circumstances allow colonization, and, in some cases, also infection by multi-drug resistant microorganisms (MDRM) [1], especially if the latter have good/strong environmental resistance in some hospital reservoir [2-4].
Many publications have reported an increased risk for colonization or infection in patients admitted to rooms where the previous patient was colonized (or infected) by an MDRM, such as methycillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus*, *Enterobacteriaceae* with extensive spectra of beta-lactamase (ESBL) or carbepenemasae [5-10]. This is mainly due to a failure in inter-patient cleaning or disinfection of these rooms that results in the non-elimination of some surface microorganisms that can then colonize or infect the next patient to use the surface [11-16]. Usually cleaning or disinfection is successful and our surface studies consistently indicate no MDRM in the rooms that had held colonized patients. Therefore, if a new patient is contaminated, it must be due to microorganisms being carried by healthcare workers who have not followed contact precautions or from microorganism reservoirs that are difficult clean during the inter-patient room cleaning/disinfection [17, 18].
Reservoirs have sometimes been seen with hydrophilic bacteria like *P. aeruginosa* or *B. cepaciae* or *Enterobacteriaceae* as KPC-*K. pneumoniae* [19] (but not among OXA-*K. pneumoniae*) or VIM-*Klebsiella oxytoca* carbepenemase (a metallo-b-lactamase that gives resistance to most antibiotics used in our hospitals). These *Klebsiella* have been detected in outbreaks in different tertiary hospitals and are associated with sink contamination; hence,

according to Lowe 2012 [2]: "Sinks should be considered as potential reservoirs when clusters of infection caused by *K. oxytoca* are investigated." However, the pathogenic mechanism explaining the transmission from this reservoir to the patient remains unclear.

A similar reservoir has not been reported for *K. pneumoniae* with carbepenemase type OXA48. Siphons in sinks can harbor biofilms, which, in addition to hampering disinfection, can facilitate survival by microorganisms. Therefore, this added factor should be considered when assessing antimicrobial actions to achieve surface bacterial removal [20].

Since we found clusters of these cases in some rooms in our hospital, we decided to investigate whether the sinks in the patient's rooms were acting as reservoirs.

## Material and methods

### A) Laboratory: in vitro studies

*Materials:*

a. Microorganisms: collected from clinical samples or weekly rectal swabs of patients from La Paz University Hospital (ICUs and Services with at least one case of OXA48-*K. pneumoniae*).
   I) Two strains of *K. pneumoniae* with OXA48-carbepenemase (strains with international dissemination such as sequence-types ST-11 and ST-405, which are involved in more than 75% of colonization cases in our hospital).
   II) Six strains of microorganisms with VIM-carbepenemase (one *K. pneumoniae*, two *K. oxytoca*, two *Enterobacter cloacae* and one *Serratia marcescens*).

b. A surface-germ-carrier (a standard-sized, easy-to-manipulate surface model [21]): rectangular glass cover-slides sized 12 x 15 mm. The number of bacteria in 10 µl of nutrient broth (after 24 h incubation at 37ºC) or on the germ-carrier contaminated with 10 µl of this broth was very similar (6.65 $\log_{10}$ vs 6.48-6.7), indicating excellent recovery by the inoculums on these germ-carriers.

c. A slime-germ-carrier (to favor slime formation): A 5-cm long brush for test tubes that has a metal center with circumferential bristles at different heights.

*Methods:*

1. Study of microbial competition between strains with OXA48 and (or) VIM carbepenemases on a slime germ-carrier.
   - Brush, with a large surface in the form of close-sitting bristles to promote the formation of slime were introduced in a diluted nutrient broth (Nutrient broth, Difco, diluted 10 times in sterile distilled water). Six tubes, each with a brush and 10 ml of this nutrient broth were prepared. In the 1st and 4th tubes one colony of OXA48-*K. pneumoniae* was introduced; in the 2nd and 5th, one colony of *S. marcescens* with VIM carbepenemase; and in the 3rd and 6th, a colony made up of both species. On the 5th day, the transfer of carrier to a new nutrient broth for the first set was interrupted, when three carriers with 5-day old colonies adhered to them, were transferred to a nutrient broth diluted 10 times and centrifuged at 2,000 rpm for two min.
   - Three 0.1 ml aliquots of the supernatant from each fifth-day carriers were extracted and plated them either directly on 4 McConkey plates, or after 1/100 or 1/10,000 dilutions to facilitate counts of colony forming colonies units (FCU). In parallel, other aliquots from these tubes were seeded (directly or diluted as above) on OXA-plates. After incubating the seeded plates at 37°C for 48 h, the FCU of the brush samples were counted. The difference between the number of FCU on McConkey and OXA-plates was estimated as the FCU-VIM recount. The second set of three tubes with individually, or jointly, seeded bacteria were transferred to new broth diluted in sterile water each day until the 10th day and then handled them the same as the first set on the 5th day, before counting surviving FCU.
2. Study of the adhesion and dissemination ability of these *K. pneumoniae* from hands to surfaces.

We cultured OXA48-*K. pneumoniae* (ST11) for 24 h in a nutrient broth at 37ºC and 10 µl of the culture was removed to contaminate finger pads of five volunteers. The pad of the thumb was rubbed over the index finger, to achieve a uniform distribution of inoculum on both fingers. After allowing the fingers to air-dry for 15 min, one surface-germ-carrier was extracted, with flame-sterilized tweezers, and placed between the two contaminated fingers. Next, the volunteers pressed their fingers for one second to contaminate the first germ carrier and then deposited it into a test tube with 5 ml of nutrient broth with 0.5 g of sterile glass beads. Immediately, each volunteer took another surface-germ carrier between the same fingers and after contaminating it for one second, deposited it in another test tube (as was done with the first germ carrier). This operation was repeated with 18 additional surface-germ-carriers. After, all volunteers applied an alcohol-solution of proven efficacy on these bacteria to their hands. In all, 20 germ-carriers were contaminated by each volunteer, and then the test tubes with the germ carriers were centrifuged at 20,000 rpm for two min. We extracted three 0.1-ml aliquots of each supernatant and plated them directly (as well as after dilutions of 1/100 to 1/10,000, for improved microbial counts) on sterile McConkey plates. These were incubated for 48 h at 37°C and then the number of FCU in each was counted.

The above was repeated using VIM-*K. pneumoniae* instead OXA48-*Klebsiella*.

### B) Clinical: Epidemiological and microbiological studies of patients and their rooms

Weekly studies on the colonization of the fecal microbiota (swabs) of all patients admitted to two areas of

our hospital (Traumatology and Oncology) were performed in the period of study (18 and 12 months, respectively), because there had been cases of infection with OXA48-*K. pneumoniae* in some of these rooms. Moreover, we took samples from the sinks in each room with a sterile swab, either directly, if the siphon could be reached without removing the drain, or after disassembling it. We sent the samples to the Microbiology Service and they were processed as if they had some from the patients. If OXA48-*K. pneumoniae* was isolated, PCR determined the strain to which it belonged. The genetic relationships between the isolates of OXA48-*K. pneumoniae* were determined by automated repetitive-sequence-based PCR using the DiversiLab® system (bioMérieux). The multilocus sequence typing (MLST) – "sequence type" – was determined according to the Institute Pasteur scheme (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html).

This helped to assess whether or not there was a relationship between the strain isolated from the siphon and those from patients admitted to the room.

All the sink siphons were changed (in the same month) in the rooms of the oncological patients. However, this was not done in the rooms of the Traumatology, because it would have required construction work that could not have been done without closing the room to new admissions. These rooms were subjected to a chemical treatment with a surface-disinfectant comprised of a chlorinated product (4,000 ppm chlorine) and anionic surfactants. This product is currently used as a surface disinfectant in our hospital. We chose it because it has an oxidant with a surfactant that can be useful in eliminating both the microorganisms in the sinks and the slime that protects them. After we had treated the sinks by pouring 5 l of this product down each drain, we collected samples from the siphon to check whether the treatment had been successful. In cases where it had not, we increased the volume of the disinfectant poured down the sink by 5 l. In one room we failed to kill bacteria with this method, so we applied heat by using steam through a steam cleaner (Lavorwash®, model Starsteam, type HP58DS-M), which has been reported to destroy slime. Subsequently, we poured another 5 l of the same disinfectant down the sink. Thereafter, we took new microbiological samples, as in previous cases, to assess the efficacy of this combined heat and chemical disinfection method.

In the following 9 months we sent weekly samples from the patients of this hospital floor to the Microbiology Service and they were processed to investigate if they had some carbepemase microorganism. If OXA48-*K. pneumoniae* was isolated, PCR determined the strain to which it belonged. Finally, we took new samples from the sink in rooms where *K. pneumoniae* with OXA48 had been isolated.

## Results

*Microbial competition:* in a media with scarce nutrient, and surfaces where slime can develop (Fig. 1), individual strains grew well, with similar increases in the number of FCU between days 5 and 10 (approximately 6 $\log_{10}$). However, in the mixed cultures (*Serratia* plus *Klebsiella*) the OXA48-*K. pneumoniae* multiplied as

**Fig. 1.** Growth of two microorganisms cultured separately or together, in a model to forming of biofilm.

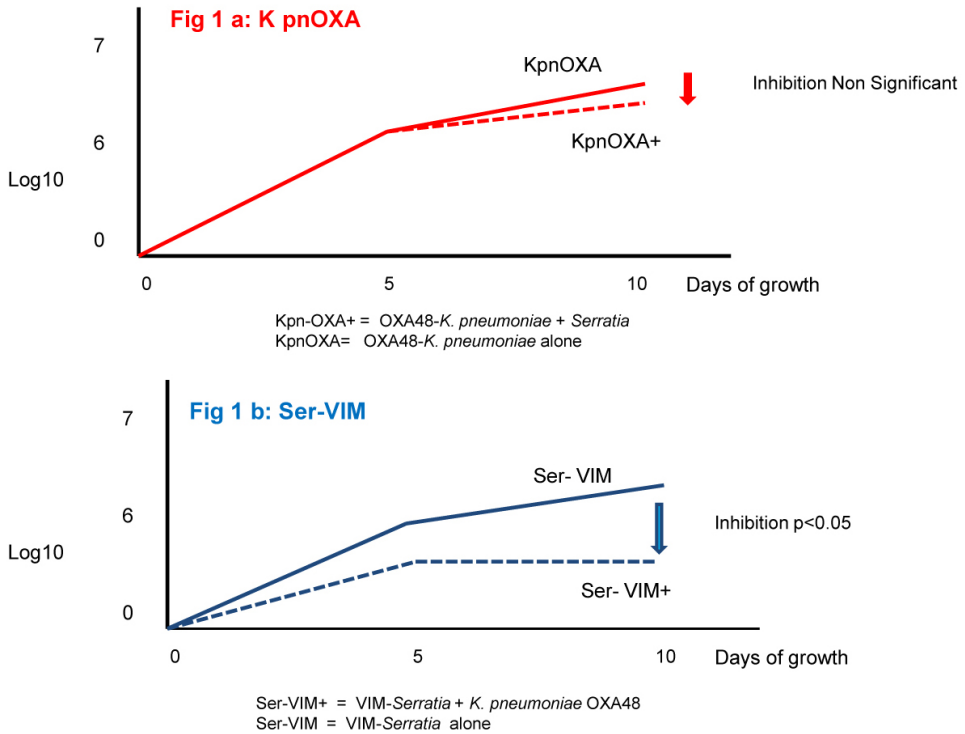
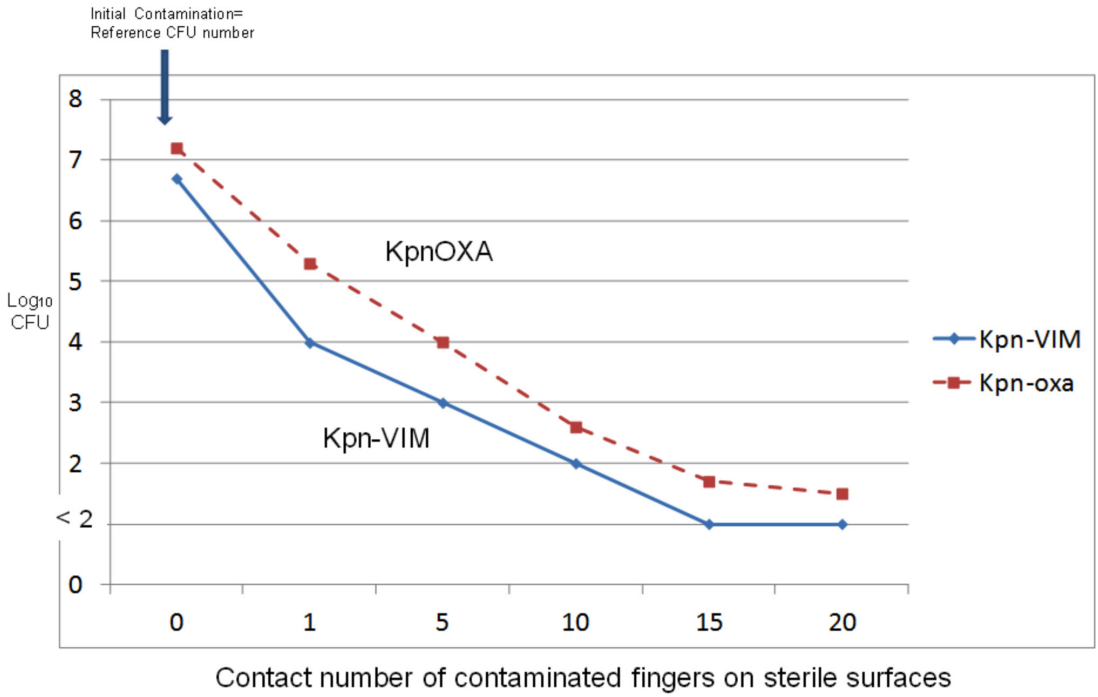


**Fig. 2.** Possibilities of dissemination of OXA or VIM *K. pneumoniae*, from human fingers (mean of CFU $log_{10}$-reduction, after successive surface contacts).




if sown alone ($p > 0.05$ = NS), whereas VIM-*Serratia* growth slowed by one decimal logarithm, and the trend was a relative decline in percentage ($p < 0.05$).

*Diffusion power:* Figure 2 shows the large diffusion capacity of hand contamination, detecting a transmission of a high number of FCU after more than 20 successive contacts. So, a single subject could contaminate several patients if the contact was made without antisepsis. There was a difference between the OXA48 and *VIM:* the slope of the line describing the transfer from hands to surfaces is lower for VIM-*K. pneumoniae* than for OXA48, implying a greater diffusion capacity from hands contaminated with OXA48 than VIM-*K. pneumoniae* (higher number of contacts with more than 3 $log_{10}$ of microorganism survival: VIM, 5 contacts and OXA 8 contacts).

The Traumatology hospitalization area was studied in two steps (Tab. I): first from July (2013) to March (2014). There were 29 patients with OXA48 microorganisms, from these, 28 were OXA48-*K pneumoniae*. All these bacteria were genotyped, except two. Moreover, OXA48 was only detected in sinks of 4 of the 21 rooms: room 1 (only in the last month, the other months it showed *Raoultella* with VIM), and room numbers 12, 18 and 20. In other rooms VIM microorganisms, such as *Citrobacter K. oxytoca*, *Enterobacter*, etc. or B-lactamase type ESBL bacteria, or microorganisms without antibiotic resistance, were detected.

If we accept contamination from the water reservoir as possible only if species and strain match in each room, this occurred in 10 of the 26 cases with OXA48-*K pneumoniae*. However, none of the siphons colonized by VIM matched the bacteria found in the patients in the same room (being different species it was not necessary to type them, see Table I). The number of studied patients was 463. The incidence of OXA48-*K. pneumoniae* in this period was 13.2% in the case of rooms with siphons positive for this bacterium and 4.7% if the siphons were negative. The OR (room with OXA48 to room without OXA48) was 3.1 (1.4-7.1; $p < 0.01$), that is, we have a suggestive coincidence (causality) between a water reservoir for specific rooms and their patients, but that is only true in the case of bacteria with OXA48, but not with VIM.

Drains were not changed in the Traumatology area because it would have required construction work. We therefore tried to treat contamination and slime with chlorine disinfectant (already used on surfaces). Pouring 5 l in the sinks of the 4 rooms, achieved success in the 12$^{th}$ room (where only *Serratia* with VIM had been detected) and the 20$^{th}$ (where *K. pneumoniae* with ESBL had been found), but rooms 1 and 18 continued to show OXA48. The application was increased to 10 l and success was obtained in room 18, but not in room 1. After treating the room 1 drain with steam under pressure, we poured 5 l of the same disinfectant and only found *Raoultella* with VIM (the bacteria that colonized this sink in the first months of the study, before it was colonized by OXA48).

The second step was following inpatients admitted to this floor during another 9 months (Fig. 3). The number of patients was 426. Cases were regularly distributed among the rooms, but strain ST11 was not found, and, practically, all cases were ST405. We compared

**Tab. I.** Summary of epidemiologic surveillance of bacteria with carbepenemases in Traumatology during 9 months (weekly cultures of patients).

| | OXA microorganisms | VIM microorganisms |
|---|---|---|
| Number of patients with colonization | 29 | 19 |
| Species and strain isolated in patients | 28 K. pneumoniae:<br>ST405: 20<br>ST11: 5<br>strain 9: 2<br>strain 6: 1<br>ND strain: 2<br>1 E. coli | 6 K. pneumoniae<br>1 S. marcescens<br>6 K. oxytoca<br>3 E. coli<br>3 E. cloacae |
| Number of sink-room with these bacteria | 4 | 12 |
| Species and strain isolated in these sinks | 3 K. pneumoniae ST405<br>1 K. pneumoniae strain 9 | 1 K. pneumoniae<br>4 S. marcescens<br>3 K. oxytoca<br>3 C. freundii<br>3 E. cloacae<br>1 R. planticola |
| Coincidence sink-patient | 10 (38%) | 0 (0%) |

this against the contamination of these siphons during this second period and the results were: rooms 1 and 18, again positive for OXA48 but rooms 12 and 20 were negative. The OR (room with OXA48 to room without OXA48: 10-8.5%) was 1.2; $p > 0.1$ non significant.

In the Oncology hospitalization area (Fig. 4), we considered patients diagnosed between April and October 2013 (34 cases, 22 with strain ST11, and 10 with strain ST405) from 259 patients. This is similar to what occurs throughout the hospital (strain ST11 is predominant). Four of the cases were clinical infections (three urinary tract infections and one septicemia). The rest were only colonization. In October the siphons were changed (because this did not require building work) and since then second period between October to Mars, with 155 studied patients), the number of OXA48 cases decreased (14); strain number 1 has been eliminated and only strain 11 remains, only as colonization.

**Fig. 3.** Spatial represention of OXA48-*K. pneumoniae* distribution by rooms in traumatology during two periods.

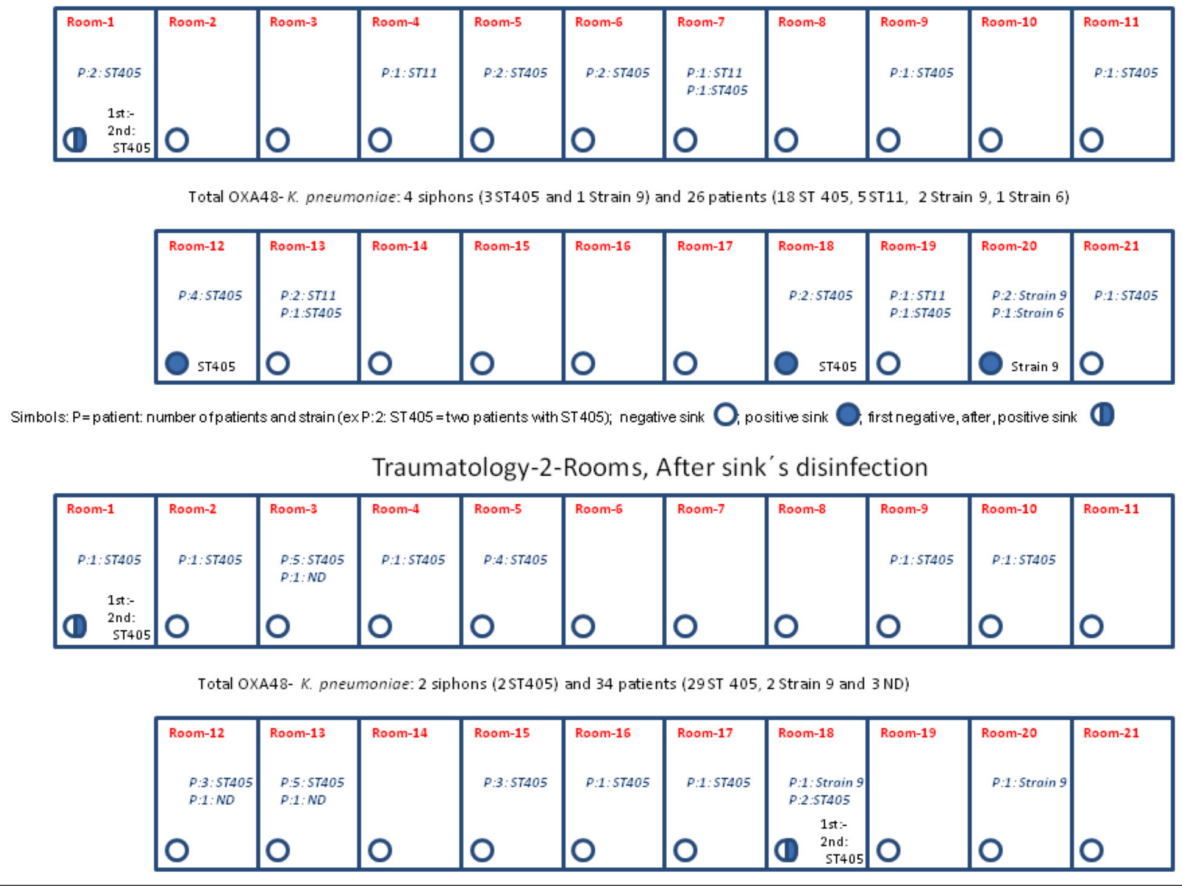
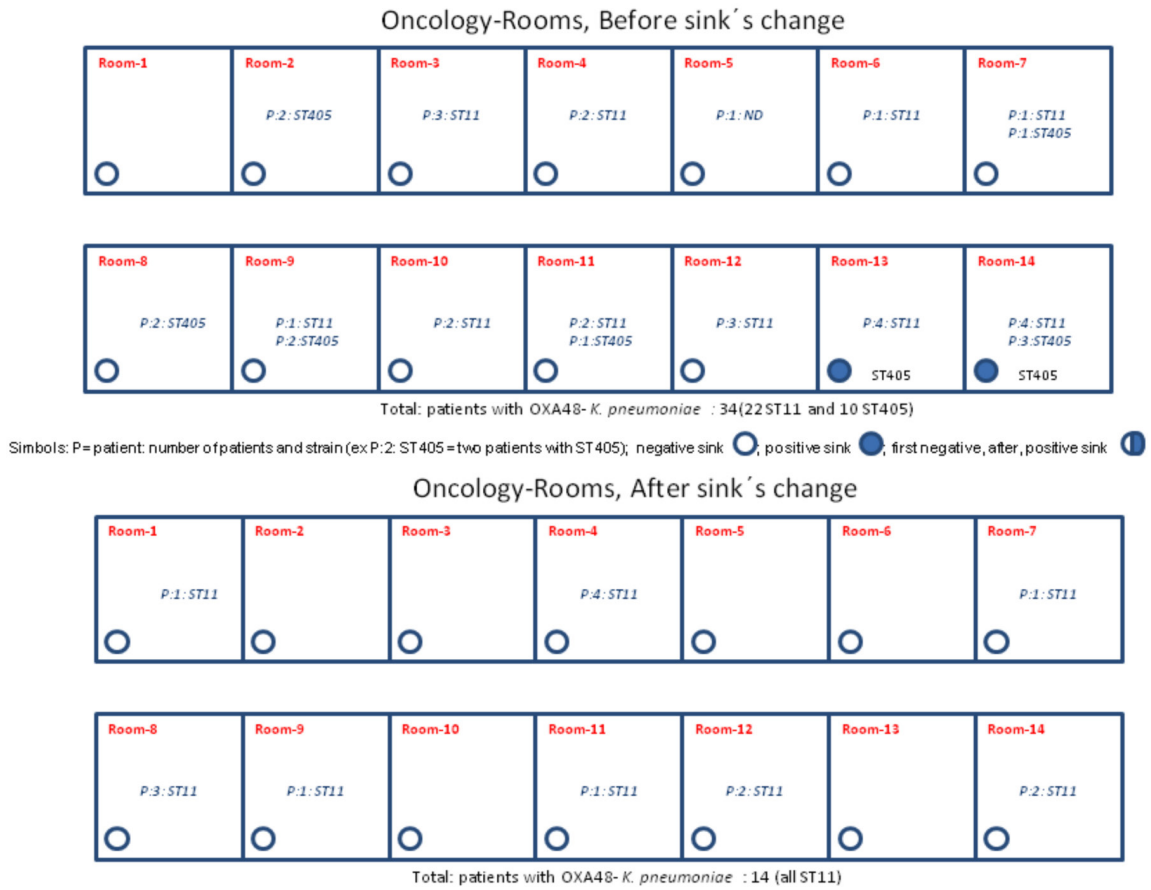


**Fig. 4.** Spatial represention of OXA48-*K. pneumoniae* distribution by rooms in oncology during two periods.




These cases predominated in rooms 13 and 14, where we detected OXA48-*K. pneumoniae* in the siphons. Before changing drains there were 28.2% in rooms 13 and 14, and 10.5% in the other rooms. The "rooms with OXA48 to room without these bacteria" OR was 3.3 (1.4-7.6; $p < 0.01$). After the change, the incidence dropped to 4.8% in rooms 13 and 14, and remained similar in the other rooms (9.7%). The relative risk in rooms with OXA/without OXA48 was 0.48 ($p > 0.1$). All these data suggest that OXA48 exposure from a contaminated sink is a probably interesting causal factor, and can be eliminated by changing the siphons without changing the other factors that continue to operate normally in the other rooms.

Lastly, the conjugation of OXA48-*K. pneumoniae* with other intestinal species of bacteria is not easy, because less than 0.5% of colonized patients by OXA-bacteria in our hospital, have two or more different species with OXA-plasmid, having been studied a grand number of rectal swabs in Oncology (2602) and Traumatology (2733) during the investigate period.

## Discussion

### Principal features

1. High possibility of microorganism diffusion from contaminated hands, which continue to transmit a large number of microorganisms after more than 10 successive contacts to surfaces, but was more intense (higher number of FCU) in OXA than VIM bacteria. These are especially important for healthcare workers, but also for the patient and his/her family.
2. Microbial competition shows that VIM bacteria are inhibited by OXA organisms.
3. In another paper [22] we have demonstrated that these bacteria have a large capacity for survival on dry surfaces (same germ-carrier as used in experiments of diffusion from the hands): VIM and OXA48 bacteria can, respectively, survive for 35 and 21 days in the environment. Klebsiella with KPC-gene, has too a grand capacity of survival in environment [19, 23] and this can be greater in humid conditions [19, 24].

These facts can explain the concentration of cases of OXA48-*K pneumoniae* in some rooms in Traumatology and Oncology, with a significant difference in risk between rooms with contaminated siphons by these bacteria and the other rooms. Risk was lowered after changing

(more reduction of risk) or disinfecting (heat plus chlorinated disinfectant, with worse results) the contaminated siphons.

However, the colonization of siphons by VIM bacteria was not related to human cases with similar microorganisms.

Normally, *Enterobacteriaceae* with carbepenemases are transmitted between patients by momentarily colonized hands of health personnel, family or patients, as reflected by the large numbers of surfaces contaminated by finger contamination (Fig. 2). But VIM-*K. pneumoniae* is less transmitted than OXA48. Perhaps the main reasons were the lower microbial adherence to surfaces or fingers by VIM-*K pneumoniae* (after the first contact, the number of FCU was low) and it is not easily transferred between patients or from contaminated surfaces to patients, probably due to changes in the capsular polysaccaride (very frequents in *Klebsiella*) [25].

Moreover, based on the above obtained risk ratios, we believe that water-born OXA48 bacterial colonization may be another risk factor for patients admitted to these rooms, but not for VIM bacterial colonization with the exception of *K. oxytoca* with VIM, with cases described in other hospitals [2, 3] but not in these Traumatology patients, because we found 6 patients with this intestinal bacteria and 3 sink colonization, but no coincidence in strains between patients and their room. Perhaps, the less growth on the biofilm due to the lower capacity for microbiological competition between microorganisms with VIM versus those with OXA48 (together with the above commented minor adherence to skin or surfaces), reduces the likelihood of patient colonization, as was found in the Traumatology Service.

Contaminated siphons must be treated by pouring a large quantity of a disinfectant down the drain to remove the biofilm [19]. This may be successful in some sinks, but when the biofilm is thicker, it requires heat together with chemical treatment. However, when siphons can be changed (without requiring building work), it is more effective and easier to change them. Nevertheless, these changes must be made without patients in the room, as the water drops during the change are contaminated with OXA48 bacteria and, if not thoroughly cleaned and disinfected, the sprinkled surfaces may allow bacteria transmission to patients.

In other rooms of our hospital where OXA48 was detected in the sinks, we used heat plus 5 l of chlorinated disinfectant and, in all of them, the OXA48 bacteria were eliminated; we now indicate this double treatment when it is not possible to change the siphons. Moreover, 7-9 months after the siphon disinfection, 50% of the sinks have again become positive to OXA48. This indicates that a systematic disinfection can be a new measure in rooms with OXA48 in their siphons (e.g. every 6 months, because the cases detected in rooms 18 and 1 were at 6 and 8 months, respectively, after being made negative by siphon disinfection). In other paper [19], recontamination of sinks after change or disinfection of siphons are described, indicating that surveillance is necessary.

Finally we present our hypothesis (a possible explanation of the observations) about the relation of water pollution in the sinks and patient colonization. The slime begins to form a base inside the siphons. Bacteria that are discharged into the sink (water from washing bedridden patients or from the toilet of not bedridden patients) adhere to this base. The most common bacteria in siphon-biofilms are VIM. But if OXA48-bacteria reach the biofilm (especially *K. pneumoniae,* due to fimbriae 1 and 3 types [26]), they will out-compete the VIM bacteria and the successive bacterial layers formed in the biofilm will only carry OXA48 bacteria. When these reach a sufficient number, they can leave the siphon by the Venturi-effect, which occurs when you open the tap, and then contaminate directly the patient during toilet (hands, face, nose, eyes) or indirectly, through the cleaning cloths used on the sink, and after, these cloths contaminated surfaces of sink, bathroom, etc. These facts allows to microorganisms be carried to patients in the same room, given their high ability to survive on surfaces and easy transfer through contaminated hands, as demonstrated in our experiments. After to get OXA48-*K. pneumoniae* to nasal or oral cavities, these bacteria can transfer its bla-OXA-plasmid (due to its grand rate of conjugation [23]) toward Klebsiella endogenous, and, in few days, all digestive tract will be contaminated with OXA48-*K. pneumoniae*, allowing be detected by rectal swab.

## Conclusions

1. The discovery of OXA48-*K. pneumoniae* in the biofilm of sinks inside hospital rooms is a result of a colonized patient's stay, and the permanence of the bacteria, as a reservoir, may contribute to the perpetuation of an outbreak in hospital patients.
2. OXA48-*K. pneumoniae* grows better than VIM-*Enterobacteriaceae* on a biofilm (example, in the siphon of a sink).
3. After contamination of a patient, family or health worker's hand, they can distribute OXA48-*K. pneumoniae* to a large number of surfaces. In the case of VIM bacteria, the dissemination is less effective.
4. Heat-chemical treatment of this biofilm (repeated every 6 months) should be regarded as one step in the strategy for controlling an outbreak of OXA48-*K. pneumoniae*, if siphon change is not possible.
5. The elimination of VIM-bacteria from sink-reservoirs by disinfection is very difficult, but the risk for colonization of the patients admitted to these rooms is low.

## Acknowledgements

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## Author contributions

RH conceived and designed the research. GR, RH and MJV performed the microbiological analysis. LR and VP-B collected the epidemiological data. RH, MS and VP-B performed the statistical analysis. RH and MS wrote the manuscript. All authors revised and approved the final manuscript
Revision of the test by a native English speaker (C. Warren).

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ORIGINAL ARTICLE

# An exception to the rule "no association between antibiotic resistance and decreased disinfectant microbicidal efficacy": Orthophthalaldehyde (OPA) and *Pseudomonas aeruginosa* isolated from ICU and paraplegic patients

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Keywords

Bactericidal effect • OPA • *P. aeruginosa* • Antibiotic-resistance

Summary

**Background**. *Antibiotic resistance and decreased susceptibility to disinfectants are not usually associated in microorganisms, but we have found an exception to this rule: P. aeruginosa versus orthophthalaldehyde (OPA).*
**Methods**. *Bactericidal effect of OPA was measured at 10 minutes on endodoncy files contaminated with an ATCC strain (control) or 206 strains of P. aeruginosa recently isolated from 206 ICU and paraplegic patients in a tertiary university hospital, in two consecutive years.*
**Results**. *Differences in bactericidal effect of OPA were found between the strains isolated each year (decreased susceptibility in the first period), but in both years the statistical differences ($p < 0.05$) were maintained according to whether the strains were "susceptible" to antibiotics, "resistant" (to one family of antibiotics) or "multi-resistant" (resistant to more than one family of antibiotics), exhibiting a reduction in their OPA susceptibility in parallel to an increase of their antibiotic resistance. In contrast, there were no differences depending on the type of sample (sputum, urine, faeces, pharynx) or of patient (paraplegic or ICU: adult, newborn, burn). Finally we selected 15 strains with an OPA effect below 3.5 $log_{10}$ at 10 minutes and repeated the study with an OPA exposure of 15 minutes. In these conditions OPA showed a total bactericidal effect on these P. aeruginosa strains.*
**Conclusions**. *There was an association between antibiotic resistance and decreased OPA susceptibility. This normally does not require an increase in disinfection time, but, for endoscope disinfection or instruments from colonized/infected patients with resistant/multiresistant P. aeruginosa, we consider it better to use 15 min of OPA. Regular tests (e.g., once every 12 months) with germ-carriers, should be performed to assess ecological changes in susceptibility to high level disinfectants and must include not only ATCC strains, but also recently isolated microorganisms with different antibiotic sensitivities (susceptible, resistant and multi-resistant).*

## Introduction

Globally, there is no association between antibiotic resistance and decreased susceptibility to disinfectants, but this may not be so with some disinfectants and microorganisms [1, 2]. Russell [3] states that low levels of biocide resistance can be detected and questions if this increased resistance could be related to domestic disinfectant use. This would first allow for a generalized selection of organisms that are more resistant to disinfectants, and these could colonize people through water and food [4]; second, an intestinal selection could occur through the use of antimicrobials. This would be better detected in the clinical setting, particularly in ICUs, where antibiotic consumption is greater [5, 6].
Complete resistance (no efficacy) to disinfectants is rare and we can only demonstrate a "decreased susceptibility" to these products. This lower susceptibility is normally related to cell wall alterations (the loss of porins or the presence of incomplete lipopolysaccharides [7]) with changes in their permeability, or to an increase in the mechanisms for expulsion of products that are harmful to the bacterium [8-10]. This decreased susceptibility is not specific to disinfectants or antibiotics [11], since both can be associated in some bacteria.
In an earlier work [12] we observed a minor susceptibility of *P. aeruginosa* to orthophthalaldehyde (OPA) that was related to antibiotic resistance; strains exhibiting resistance to one or more than one antibiotic family ("resistant or multi-resistant" strains) also had a decreased susceptibility to the disinfectant. But our previous results [12] also have demonstrated an increase of OPA effectivity without changes in antibiotic resistance, after aging the same strains of *P. aeruginosa*. This indicated that the two mechanisms were independent, and were only "concurrent", in recently isolated microorganisms from the patients.

Last, *P. aeruginosa* is a very frequent microorganism in infections of ICU or paraplegic patients and it normally, shows resistance to antibiotics. Moreover, these bacteria produce reiterated contamination of endoscopes, probably due to their ability to form a biofilm, and this can produce failures in high level disinfection processes [13-16]. Consequently, *P. aeruginosa* from these patients can be a good model of interaction between inadequate disinfection and antibiotic resistance.

The objective of this study was to confirm the association between a lower susceptibility to OPA and greater antibiotic resistance in *P. aeruginosa* with a larger sample of microorganisms, including not only resistant [12], but also susceptible strains, and, as well, to study if these conditions are associated to the type of sample used (source of microorganism, type of patient) or to resistance to a specific antibiotic.

## Materials and methods

### Materials

- Disinfectants: OPA: 0.55% orthophthalaldehyde (Johnson & Johnson, Irvine, CA, USA).
- Microorganisms: *P. aeruginosa* ATCC 27853, and 206 *P. aeruginosa* strains isolated from patients during two consecutive years.
- Germ-carrier: Number 25, endodoncy files (difficult to disinfect; these pieces, used in endodoncy work, have a rough metallic surface with a rough plastic end).
- Glass beads (0.25 mm in diameter).
- Inhibitor of disinfectant action: Todd Hewitt broth (Difco) plus 6% (w/v) Tween 80, 0.5% (w/v) sodium bisulfite, and 0.5% (w/v) sodium thiosulfate.
- All culture media and Tween 80 used in this research were purchased from the Madrid Autonomous University Foundation (*Fundación Universidad Autónoma de Madrid, FUAM*).

### Methods

During two consecutive years (2011-2012), 206 samples (103 per year) were isolated from 206 different patients admitted to the different ICUs of Hospital La Paz (General, Burn and Newborn Units), as well as from urine or decubitus ulcer samples taken within the first three months after injury from paraplegic patients admitted to hospital. The sample (only one per patient) was the first *P. aeruginosa* isolated during their hospital stay, and the bacterial antibiotic susceptibility or resistance was recorded.

In the first week after isolation of these strains, we studied the bactericidal effect of OPA, using a method described in earlier studies [2, 12, 17] and summarized below:

#### Determination of bactericidal effect of a disinfectant employing a metal/plastic germ-carrier (number 25 endodoncy files)

Endodoncy files (an excellent model of rough-carrier), were contaminated with a suspension of one strain of these *P. aeruginosa* ($10^8$ CFU/ml) by immersion for one hour before being left to dry (15 minutes) on a slanted sterile surface (Petri dish with no culture medium). After drying, the germ-carrier was placed in a tube with 7 ml of the disinfectant for 10 minutes. The carrier was then removed and placed in another tube containing 7 ml of inhibitor with 0.5 g glass beads (1 mm in diameter) and vortexed for one minute at 1000 rpm. Finally, 0.1 ml of the supernatant was cultured on Mueller-Hinton plates and incubated at 37ºC for 24 hours in order to count the number of microorganisms surviving after exposure to the disinfectant. The CFU counted were compared with these obtained for the control (using the same method but introducing the germ-carrier in sterile distilled water instead of disinfectant). The assay was performed for all microorganisms described in the Materials section.

The cut point for considering "reduced OPA susceptibility" was a $\log_{10}$ reduction of less than 3.5 [17] (equivalent to below 5 $\log_{10}$ when using the test involved glass germ-carriers in the EN-test, as described elsewhere [12]). Both cut points (according to their test) indicate an inadequate disinfection.

Finally, of 15 randomized strains, among all those exhibiting a bactericidal effect of $< 3.5$ $\log_{10}$ after 10 minutes of OPA exposure, we repeated the test with a 15 minute exposure.

#### Antibiotype

The antibiotype was obtained on the same day of the bactericidal effect with each *P. aeruginosa* strain. The method used was Kirby-Bauer. The cut point for being considered resistant was according to CLSI, 2007.

#### Statistical analysis

For the statistical study, a $> 5.5$ $\log_{10}$ reduction (experiments in which there were no surviving *P. aeruginosa* CFU) was considered as "5.5" $\log_{10}$.

Demonstration of significant differences between OPA susceptibility ($\log_{10}$ reduction) and antibiotic susceptibility according to type of microorganism source was done using an analysis of variance (ANOVA) or a Mann-Whitney U test and Kruskal-Wallis test, since nonparametric tests were used for all samples lacking a normal distribution.

Finally we have performed a multivariable analysis by logistic regression, taking as dependent variable the $\log_{10}$ reduction of *P. aeruginosa* ($< 3.5$ log = 1 and $\geq 3.5 = 0$) and as independent variables, type of patients, source of strains, year and antibiotype (classified into susceptible, resistant and multi-resistant or by specific antibiotics too).

## Results

Patient distribution was: general ICU 75.7%, neonatal ICU 12.1%, burn unit 4.8% and paraplegics 6.8%.

The source of the 206 *P. aeruginosa* isolates from the 206 patients was as follows: pharynx or sputum 70.1%,

**Tab. I.** $log_{10}$ reduction of 207 *P. aeruginosa* (206, isolates from 206 patients and one ATCC strain), on germ-carrier, according to antibiotic resistance, after 10 minutes of exposure to OPA.

| $log_{10}$ reduction by OPA | | | | |
|---|---|---|---|---|
| | Mean | Standard | 50-Percentile | p |
| | | Error | | |
| **Antibiotic susceptibility** | | | | |
| ATCC* | 5.5 | 0** | 5.5 | |
| | | | | < 0.01 |
| Susceptible | 4.9 | 1.1 | 5.5 | |
| | | | | < 0.01 |
| Resistant | 4.46 | 0.97 | 4.6 | |
| | | | | < 0.01 |
| Multi resistant | 3.97 | 1.1 | 3.88 | |
| * Repetition of 10 tests with the same ATCC P. aeruginosa: control strain; ** There was no variability among the different experiments, because > 5.5 log10 was always obtained (recorded in the statistical analysis as 5.5). | | | | |

**Tab. II.** $log_{10}$ reduction of 207 *P. aeruginosa* (206, isolates from 206 patients and one ATCC strain), on germ-carrier, after 10 minutes of exposure to OPA, according to the origin of the bacteria.

| $log_{10}$ reduction by OPA | | | | |
|---|---|---|---|---|
| | Mean | Standard | 50-Percentile | p |
| | | Error | | |
| **Type of sample** | | | | |
| Pharynx/sputum | 4.2 | 1.1 | 4.1 | |
| | | | | NS |
| Urine/faeces | 4.3 | 1.2 | 4.4 | |
| | | | | NS |
| Nasal | 4.4 | 1.1 | 4.7 | |
| | | | | NS |
| Other | 5.1 | 0.75 | 4.8 | |
| **Type of patient** | | | | |
| General ICU | 4.3 | 1.1 | 4.4 | |
| | | | | NS |
| Neonate ICU | 4.4 | 1.1 | 4.9 | |
| | | | | NS |
| Burn unit | 4.6 | 0.75 | 4.5 | |
| | | | | NS |
| Paraplegics | 4.1 | 1.05 | 3.95 | |
| | | | | |

urine or faeces 15%, nose 8.9%, and other 6% (i.e., burn, decubitus ulcer or central venous catheter insertion site). These 206 *P. aeruginosa* were distributed into three groups based on antibiotic susceptibility: "susceptible" (only natural resistance) 21.8%, "resistant" (to one antibiotic family – independently of what it was – in addition to natural resistance) 34%, and "multi-resistant" (resistant to two or more antibiotic families, in addition to natural resistance) 44.2%.

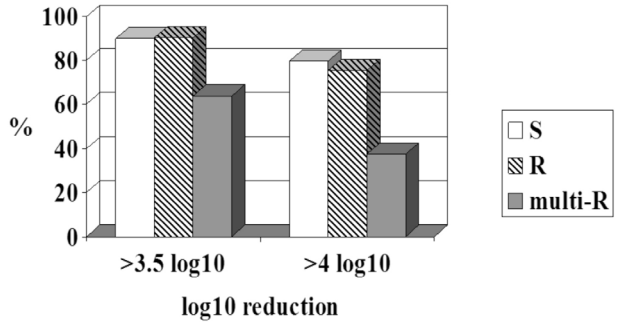
The logarithmic reduction originated by OPA on *P. aeruginosa* strains was distributed according to the above variables. Last we included the year of diagnosis.

Tables I and II showed that antibiotic susceptibility is the only parameter that significantly ($p < 0.001$) differentiated the *P. aeruginosa* strains. Moreover, the ATCC *P. aeruginosa* strain was also different from the antibiotic-susceptible strains, because the ATCC strain was fully susceptible to OPA (0 survivors in all experiments).

On the one hand, Figure 1 shows the overall frequency of strains on which OPA had a bactericidal effect > 3.5 $log_{10}$ (the optimum threshold in this test) or > 4 $log_{10}$ (considered here as "great efficacy"). It can be seen that 90% of antibiotic susceptible or resistant *P. aeruginosa* strains reached the 3.5 $log_{10}$ reduction with OPA while this product only had a similar bactericidal level in two-thirds of the multi-resistant strains, with significant differences between the susceptible, resistant and multi-resistant strains. However, great efficacy (> 4 $log_{10}$ reduction), was still achieved in 79.5% of the susceptible and 75.4% of the resistant strains but in only one-third of the multi-resistant strains. In both thresholds, there were significant differences between the OPA's effect against susceptible or resistant strains versus the effect in multi-resistant strains.

In 15 randomized strains (among all those exhibiting a bactericidal effect of < 3.5 $log_{10}$ after 10 minutes) were exposed to OPA during 15 minutes. We obtained a complete destruction of all the microorganisms, suggesting that even in the worst case scenario (and using more resistant strains and complex instruments), a complete

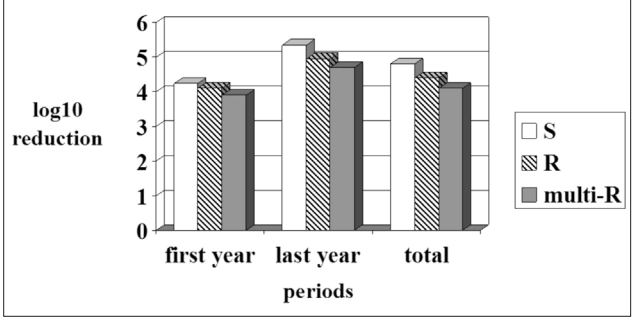
Fig. 1. Bactericidal effect of OPA against 206 *P. aeruginosa* strains, by antibiotype.




destruction of the microbial inocula could be achieved by simply prolonging exposure by 5 minutes.

Last, Figure 2 shows the existence of differences between the two studied years. Thus, in the first, *P. aeruginosa* isolates were on average more resistant to OPA than in the second year, but the differences between susceptible, resistant and multi-resistant strains were maintained in both periods. When all strains were jointly assessed, (or when the strains from the first year were considered only) a statistical association was found between resistance to imipenem or two aminoglycosides (gentamycin and tobramycin) and a bactericidal effect below the optimum threshold of 3.5 $\log_{10}$. However this did not occur in the second year, since the *P. aeruginosa* isolates were much more susceptible to OPA, and only a small proportion fell below the threshold, whereas the resistance to these 3 antibiotics remained virtually unchanged. Therefore, this antibiotic resistance to imipenem and aminoglycosides cannot be generalized as a predictive antibiotype marker for a decreased/weaker bactericidal effect. No other antibiotype was associated with a decreased bactericidal effect (taking strains resistant to 1-5 antibiotics in all possible combinations, for example: amikacin + imipenem, amikacin + ceftazidime, amicacin + fosfomycin, amikacin + imipenem + ceftazidime + fosfomycin, etc.). Multivariable logistic regression, taking as dependent variable the $\log_{10}$ reduction of *P. aeruginosa* and as independent variables, type of patients, source of strains, year and antibiotype, did not show a good fit.

Fig. 2. Bactericidal effect of OPA against 206 *P. aeruginosa* strains, by time and antibiotype.




## Discussion

The availability of a large number of samples with different antibiotic susceptibilities allowed us to adequately assess the association between *P. aeruginosa* antibiotic resistance and decreased susceptibility to a disinfectant (in this case OPA) from a statistical viewpoint (N-dependent [18]).

ATCC strains are helpful in homogenizing the results obtained in different laboratories, but they are not good predictors of the true performance of a disinfectant in the clinical setting, since these strains frequently show a complete destruction of the inocula (i.e., maximum susceptibility), unlike autoctonous strains [12, 17] (Tab. I). These considerations indicate a need to add to tests using ATCC strains other tests with strains that have been recently isolated from patients (better in the first week, with no more than a single culture passage [12]). When possible, not only antibiotic-susceptible but also resistant and multi-resistant strains should be included in these tests.

It is noteworthy that changes were also seen when the bactericidal effects were compared using microorganisms from both consecutive years (mean $\log_{10}$ reduction: $3.6 \pm 0.7$ in the first, versus $4.8 \pm 0.8$ in the second year; $p < 0.01$), and these differences persisted after stratification according to antibiotic susceptibility (Fig. 2). Another difference was also noted in these two years: the lack of an association between resistance to imipenem, gentamycin and tobramycin, and a bactericidal effect below the optimum threshold of 3.5 $\log_{10}$ in the second year. That does not allow generalization of these markers as indicators for a need to increase the OPA disinfection time.

These differences are therefore probably not due to methodological changes (method, laboratory, microbiologist, control strains, inhibitor of disinfection, etc.) because they were the same in all experiments in both periods. The patient-sources for *P. aeruginosa* were similar too. We therefore believe that the reason could be the modifications in the dominant strains in the hospital caused by antibiotic use or environmental changes (home disinfectants or antibiotherapy). This should be taken into account, and routine studies of the bactericidal efficacy of disinfectants used should be conducted to assess the need to extend disinfectant exposure time.

Implications in daily practice:

- A warning of "*P. aeruginosa* R or multi-R" should be included in the medical records of all patients colonized or infected by these microorganisms, so that the reusable instruments are disinfected with an increased OPA exposure time.
- Since overall almost a third of multi-resistant strains experience a bactericidal effect from OPA that is lower than 3.5 $\log_{10}$ (considered the threshold value of the test [12]), it could be advisable to prolong ex-

posure time to the disinfectant from 10 to 15 minutes, as with this latter time even the most resistant strains are totally destroyed. This exposure-time in endoscope disinfection with OPA is closer to USA recommendations (12 min) than European ones (5 min) [19].

- The effectiveness of OPA disinfection should be compared between instruments used in patients with resistant or multi-resistant *P. aeruginosa*. This is especially important for endoscopes, since the persistence of *P. aeruginosa*, after disinfection, means an increased risk of infection for the next patient undergoing this technique. The ideal sample in these cases is the rinse water of the equipment, after disinfection with OPA.
  However, the risk of disinfection failure is very low in any case, since if thorough washing with an enzymatic detergent is carried out prior to disinfection, the inoculum to be dealt with by the disinfectant is at the most 3.1-4.3 $\log_{10}$ on average in the case of endoscopes [20, 21]; moreover, after disinfection, supplementary rinsing will also help remove any remaining microorganisms. Thus, at the end of the process no residual microorganisms are likely to be isolated, as seen in the serial controls we perform on endoscopy equipment once a month in our hospital, even when disinfectants less potent than OPA were used [17]. However, it is necessary to maximize caution to ensure the high quality disinfection demanded by modern hospitals, and the conditions of the process should be adapted to the patients e.g. increase the disinfection time for a colonoscope, from a patient with antibiotic-resistant microbiota, to 15 min.
- We advise at least one study every 12 months using not only ATCC strains but also recently isolated bacteria on a complex germ-carrier (like an endodoncy file), to help evaluate the necessity of increasing disinfectant exposure time.

Limitations:

- This design only includes two years of *P. aeruginosa* sampling. It does not allow us to explain the cause of differences in OPA susceptibility when the patients and antibiotic use in these ICUs were similar. It would be interesting to study more years to understand the cause of these changes.
- Despite the large number of strains studied, our results did not obtain any antibiotype that would serve as a marker of reduced OPA efficacy. We only know that "resistant" or "multi-resistant" *P. aeruginosa* can produce a failure in disinfection when OPA is used, which is less useful in daily practice.
- We have not studied patients at digestive care units. This can be a problem, given that endoscopic techniques are used more frequently to treat these patients. However, we have found that the selection of resistant or multi-resistant *P. aeruginosa* among them is very low because they are less frequently treated with antibiotics than patients in UVIs, burned or paraplegic patients.
- In order to increase the homogeneity of the sample, only the first strain of *P. aeruginosa* isolated in each patient was included in the study. Unfortunately, this reduces our ability to study if the evolution of antibiotic resistance affects the susceptibility of microorganism to OPA.

## Conclusions

An association exists between antibiotic resistance and decreased susceptibility to OPA for *P. aeruginosa*, however, in practical terms the reduction in efficacy normally does not imply an increase in disinfection time, except in flexible endoscope disinfection, where 15 min is advisable, regardless of their colonization or infection with *P. aeruginosa*.

As a precaution, reusable instruments from patients colonized or infected with resistant or multi-resistant *P. aeruginosa* should be treated with 15 minutes of OPA. This must be recorded in the clinical history of these patients.

In the cases of colonization or infection by resistant or multi-resistant *P. aeruginosa*, the efficacy of disinfection should be evaluated (for example, by sending to the Microbiology laboratory a sample of the rinse water of the endoscope after disinfection with OPA).

Regular tests (e.g., once every 12 months) should be performed to assess ecological changes in susceptibility to OPA by *P. aeruginosa* colonizing or infecting patients, in order to detect changes in strain susceptibility and to be able to recommend an increase in disinfectant time (e.g. 5-10 to 15 minutes) when necessary.

Any evaluation of the efficacy of OPA should include not only ATCC strains but also recently isolated autoctonous microorganisms with different antibiotic sensitivities (susceptible, resistant and multi-resistant), since such characteristics may condition reduced product susceptibility.

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## Authors' contributions

RH conceived and designed the research. IH, RH and MJV performed the microbiological and the statistical analysis. RH wrote the manuscript. All authors revised and approved the final manuscript.

Revision of the test by a native English speaker (C. Warren).

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ORIGINAL ARTICLE

# Water and air ozone treatment as an alternative sanitizing technology

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Keywords

Water and air ozone treatment • Alternative sanitizing technology

Summary

**Aims.** *We investigated the effectiveness of ozone (aqueous and gaseous) treatment as an alternative sanitizing technology to common conventional disinfectants in reducing the microbial contamination of both water and air.*
**Methods.** *Ozone was added for 20 minutes to a well-defined volume of water and air by the system named "Ozonomatic®". The effectiveness of ozonation was determined by counting CFU/m$^3$ or ml of bacteria present in samples of air or water collected before ($T_0$) and after ($T_1$) the addition of ozone and comparing the microbial load of different bacteria present in ozonized and non-ozonized samples.*
**Results.** *When the ozonisation equipment was located at 30 cm from the surface of the water in the bath tub in which the bacteria investigated were inoculated, the treatment was able to reduce the total microbial load present in the aerosol by 70.4% at a temperature of 36°C for 48 hours. Conversely, at 22°C for 5 days, only a modest decrease (9.1%) was observed. Escherichia coli and Pseudomonas aeruginosa were completely eliminated. A 93.9% reduction was observed for Staphylococcus aureus, followed by Streptococcus faecalis (25.9%). The addition of ozone to water was able to almost eliminate Staphylococcus aureus (98.9% reduction) and also to exert a strong impact on Legionella pneumophila (87.5% reduction). Streptococcus faecalis and Pseudomonas aeruginosa showed a decrease of 64.2% and 57.4%, respectively. Conversely, only a 26.4% reduction was observed for the bacterium Escherichia coli. This study showed that the addition of ozone in the air exerted a modest reduction on microbial load at 36°C, whereas no effect was observed at 22°C.*
**Conclusions.** *Aqueous and gaseous ozone treatments were effective against microbial contaminants, reducing the CFU of the microorganisms studied. These results confirm the efficacy of the ozone disinfection treatment of both water and air; particularly, it constitutes an extremely promising alternative, allowing the possibility to reuse contaminated water.*

## Introduction

Alternative disinfection methods, such as gaseous disinfectant technologies, have recently been introduced into the market; these constitute an additional, efficient means to manual disinfection [1-3] or, in the case of water disinfection, a valid substitute for chlorine, enabling water to be reused (i.e. water reconditioning) [4, 5]. Chlorine is the most widely used commercial sanitizing agent, and is added to the water used for washing vegetables and fruit. However, its use in food applications is associated with various problems, such as the production of several carcinogenic disinfection by-products (DBP) [6], including trihalomethanes and haloacetic acids, derived from the reaction between chlorine and organic material [7, 8]. This concern has prompted some European countries to ban its use for washing organic produce [9, 10]. Furthermore, it has been demonstrated that a gaseous sanitizer (ozone) has a greater disinfectant ability than a liquid sanitizer, owing to its uniform distribution and penetration. Indeed, gaseous sanitizers display a four-fold higher diffusivity [11]. The choice of the appropriate sanitizer depends on the processing limitations, including the residual disinfectant needed to achieve sufficient disinfection. In the water used for washing freshly cut products, hydrogen peroxide ($H_2O_2$), organic acids, US, and ultraviolet (UV) irradiation are not recommended [12]. In the literature, the efficiency of a sanitizer is currently determined by evaluating the microbial reduction, investigating the process of decontamination and, to a lesser extent, assessing the prevention of cross-contamination [12].
Among the gaseous sanitizers investigated in recent years, such as ozone, chlorine dioxide ($ClO_2$), and cold plasma, ozone has proved the most effective [13], being a powerful oxidant for water treatment, after the hydroxyl radical. Conversely, chloramines are the least efficient. Moreover, they are not recommended for primary disinfection, but for secondary water disinfection, since they react more slowly than chlorine and persist for a longer time in distribution systems [13]. Compared with chlorine, ozone needs a lower concentration and shorter contact time in order to exert its disinfectant effect [14]. Ozone aerosolization could constitute an effective alternative antimicrobial delivery system, as it is able to penetrate into all surface irregularities and is applicable to a wide antimicrobial spectrum [11]. Furthermore, water treatment through the addition of ozone could maximize

water reusability [14]. Owing to its short half-life, its toxicity and reactivity, ozone must be produced on-site, where it reacts principally with carbon-carbon double bonds, activated aromatic structures, and non-protonated amines. Ozone reacts more slowly with fatty acids and carbohydrates, while it reacts faster with proteins, amines, amino acids, nucleic acids, and protein functional groups [12].
The aim of the present study was to evaluate the ability of the ozonised hydro massage system supplied by Ozonomatic® to reduce the bacterial load present in air and in water.

## Materials and methods

### Growth of bacteria in broth cultures

*Staphylococcus aureus* (*S. aureus*) (ATCC 13150), *Pseudomonas aeruginosa (P. aeruginosa)* (ATCC 27853), *Escherichia coli* (*E. coli*) (ATCC 25922) and *Streptococcus faecalis* (*S. faecalis)* (ATCC 10541) were obtained from LGC Standards (Sesto San Giovanni, Milan, Italy). A suspension of *Penicillium*-type mycetes had previously been isolated in the laboratory. The growth medium used were: Tryptone Soya Agar (P05012A), Brain Heart Infusion (BHI) Broth (CM225), Mannitol Salt Agar (CM85), Cetrimide Agar (Sigma-Aldrich Chemical C, St Louis, MO, USA), Sabouraud dextrose Agar (CM41), Endo Agar Base Oxoid (CM0479), and Slanetz & Bartley Medium (CM0377, ThermoScientific). With the exception of Cetrimide Agar and Slanetz & Bartley Medium, the growth media were obtained from Oxoid (Wesel, Germany) unless otherwise specified. Slabs with a diameter of 55 mm and sterile cellulose acetate membrane filters were provided by Sartorius (Italy).
The lyophilized growth media were re-hydrated and sterilised in accordance with the producers' instructions. They were then dispensed on Surfair-type slabs with a diameter of 55 mm. Different media were used according to the bacteria to be isolated. Specifically, Tryptone Soya Agar was used in the search for mesofila at 36°C; Mannitol Salt Agar for *S. aureus*; Centrimide agar for *P. aeruginosa* Endo Agar Base for *E. coli*; Slanetz & Bartley Medium for *S. faecalis*; and Sabouraud dextrose Agar for mycetes belonging to the *Penicillium* type. The BHI broth was dispensed in glass test tubes as growth medium. For every above-mentioned growth medium, tests of sterility and fertility were carried out.
Growth in BHI broth was investigated for all the bacteria (*E. coli*, *S. aureus*, *P. aeruginosa*, *S. faecalis*), whereas for suspensions of mycetes (*Penicillium*-type) NaCl solution was used.

### Generation of experimental biofilms to evaluate microbiological control exerted in water by ozone

To generate an experimental biofilm of each bacterium, samples from each broth culture were added to the water in a bath tub. The tub contained 20 liters of distilled water at a temperature of 37°C and was located in a room measuring about 85 $m^3$.
After centrifugation for 20 minutes at 2,500 rpm, the supernatant was removed and 9 parts of HCl-KCl were added. After resuspension, 0.1 ml of the sample was seeded onto plates containing the specific medium for *Legionella* (Legionella CYE Agar Base). It was then dispensed onto slabs with a diameter of 90 mm after being supplemented with Legionella Growth Supplement and Legionella MWY Selective Supplement. The samples collected were incubated at 36°C in a humidified atmosphere for 10 days, with readings being taken daily.
To determine the total microbial load at 36°C, 1 ml of mixed cultivation ground of *E. coli*, *S. aureus*, *P. aeruginosa*, *S. faecalis* and *Legionella pneumophila* (*L. pneumophila*) was used to contaminate the water; to determine the total microbial load at 22°C, 1 ml of a suspension of *Penicillium*-type mycetes was used. At the time of inoculation into the bath tub, each inoculum had a minimum concentration not lower than $10^5$ CFU/ml (colony-forming units per milliliter) for mycetes and $10^7$ CFU/ml for all the bacteria. After shaking, one 10 ml and one 5 ml sample of water were collected and identified as $T_0$. These were filtered by cellulose acetate membrane filters with porosity of 0.45 μm, in order to not remove the microorganism of interest, and a diameter of 55 mm; these filters were located on the surface of the growth medium. The slabs for *E. coli*, *S. aureus*, *P. aeruginosa* and mesofila were incubated at 36°C for 48 hours, while mycetes were incubated for 5 days at 22°C. The microbial load was measured 5 times in both the 5 ml and 10 ml samples, and a mean value was calculated from the means of each set of samples. The microbial load was expressed as CFU/ml.

### Evaluation of the microbiological control exerted in water by an ozonized hydromassage system produced by Ozonomatic®

After the experimental contamination of the water by inoculating it with the bacterial suspensions, the water was exposed to ozone for 20 minutes; 5 ml of water was then collected and identified as "$T_1$". The analysis was performed in the same way as for samples collected at $T_0$. In the case of *L. pneumophila*, the samples of water collected at $T_0$ and $T_1$ were centrifuged at 2,500 rpm for 20 minutes, in order to concentrate the bacteria. The upper and lower float layers present in the test tube containing 9 parts of HCI-KCI tampon were re-suspended and 0.1 ml of each sample was seeded onto slabs containing specific medium. The samples were incubated for 10 days at 36°C in a humidified atmosphere; during the period of incubation, readings were taken daily.

### Microbiological control exerted in air by ozone

This part of the study was conducted in two phases with different aims. The first phase aimed to evaluate the possible "total microbial reduction" in the air in the room where the ozonization equipment was located; the sec-

ond phase aimed to evaluate the possible "reduction of each single microorganism" in the air.
Specifically, in the first phase, the "Microflow 60" equipment was set up in order to aspirate a volume of 180 litres of air. The apparatus was located at distance of 1m from the bath tub and at a height of 1.5 m from the floor. The first aspiration was carried out in order to measure the bacterial and mycotic loads present in the air before the action of the hydro-massage ($T_0$); the second aspiration was carried out 20 minutes after the production of ozone ($T_1$).
In the second set of experiments, the "Microflow 60" was positioned 30 cm from the surface of the water in the bath tub, which had previously been filled with 20 litres of water at a temperature of 37°C. A volume of 180 litres of air was aspirated after the addition of BHI broth culture containing *E. coli*, *S. aureus*, *P. aeruginosa*, and *S. faecalis*. A further 180 litre volume was aspirated after the addition of a suspension containing all the above-mentioned bacteria and a suspension of mycetes. Air samples were collected at $T_0$ and after 20 minutes of ozone treatment ($T_1$).
The samples collected were incubated at 36°C for 48 hours for the investigation of *E. coli*, *S. aureus*, *P. aeruginosa* and *S. faecalis* and of the total microbial load, and at 22°C for 5 days for mycetes.

## Results

### Evaluation of the antimicrobial effect of the ozonized bath produced by Ozonomatic® on water

Among the microorganisms investigated, *S. aureus* displayed the greatest reduction (98.9%) after ozonization treatment, being almost completely eliminated. Ozonization also exerted a strong impact on *L. pneumophila* (87.5% reduction). Regarding *S. faecalis* and *P. aeruginosa*, the addition of ozone to water was able to eliminate more than half of the microbial cells, obtaining a reduction of 64.2% and 57.4%, respectively. Conversely, only a 26.4% reduction in the bacterium *E. coli* was observed. A slight reduction (16.6%) was seen in mycetes, incubated at 22°C.

**Tab. I.** Total microbial load present in the water at 36°C and 22°C, and microbial load of each bacterium measured before ($T_0$) and after ozonization treatment ($T_1$).

| | $T_0$ CFU/ml | $T_1$ CFU/ml | Microbial load reduction (%) |
|---|---|---|---|
| Microbial Load at 36°C | 151 | 43 | 71.5 |
| Microbial Load at 22°C | 12 | 10 | 16.7 |
| *E. coli* | 87 | 64 | 26.4 |
| *P. aeruginosa* | 244 | 104 | 57.4 |
| *S. aureus* | 377 | 4 | 98.9 |
| *S. faecalis* | 162 | 58 | 64.2 |
| *L. pneumophila* | 495 | 62 | 87.5 |

The total microbial load measured at 36°C revealed a 71.5% diminution; this is in line with all the percentages found for the individual microorganisms, constituted by the totality of the above-mentioned bacteria. At 22°C, a smaller microbial reduction was observed (16.7%) (Tab. I).

### Evaluation of the antimicrobial effect of ozone produced by Ozonomatic® on the air

The antimicrobial effect produced by ozone on the air in the environment where the bath tub and the ozonizing equipment were situated was evaluated by comparing the total microbial load measured at $T_0$ (no ozone treatment) and $T_1$ (ozone treatment). The total microbial load (*E. coli, P. aeruginosa, S. aureus* and *S. faecalis)* measured at 36°C and 22°C proved to be low at both temperatures (26 CFU/m$^3$ and 8 CFU/m$^3$, respectively) before ozone treatment. Consequently, ozone treatment did not significantly reduce the bacterial load, a modest reduction from 26 CFU/m$^3$ to 23 CFU/m$^3$ (11.5%) being observed at 36°C. No reduction was observed at 22°C (0%) (Tab. II).
When the ozonization equipment was placed 30 cm from the surface of the water in the bath tub, ozone treatment was able to reduce the total microbial load present in the aerosol by 70.4% at a temperature of 36°C. Conversely, at 22°C only a modest decrease (9.1%) was observed.
Ozonization was able to completely eliminate the microbial loads of both *E. coli* and *P. aeruginosa* (100% reduction). A reduction of 93.9% was observed for *S. aureus*. Regarding *S. faecalis*, a smaller reduction was seen (25.9%) (Tab. III).

**Tab. II.** Microbial load present in the air before ($T_0$) and after ozonization treatment ($T_1$) at 36°C and 22°C.

| | $T_0$ CFU/m$^3$ | $T_1$ CFU/m$^3$ | Microbial load reduction (%) |
|---|---|---|---|
| Microbial Load at 36°C | 26 | 23 | 11.5 |
| Microbial Load at 22°C | 8 | 8 | 0 |

**Tab. III.** Microbial load present in the air collected at 30 cm from the water surface contained in the bath tub where the ozone equipment was located, measured before ($T_0$) and after ozonization treatment ($T_1$) at 36°C and 22°C.

| | $T_0$ CFU/m$^3$ | $T_1$ CFU/m$^3$ | Microbial load reduction (%) |
|---|---|---|---|
| Microbial Load at 36°C | 655 | 194 | 70.4 |
| Microbial Load at 22°C | 33 | 30 | 9.1 |
| *E. coli* | 378 | 0 | 100 |
| *P. aeruginosa* | 233 | 0 | 100 |
| *S. aureus* | 5,955 | 361 | 93.9 |
| *S. faecalis* | 2,400 | 1,778 | 25.9 |

## Discussion

This study reproduced a situation of water contamination due to microorganisms naturally present in the environment and in human organisms.
The search for *L. pneumophila* was conducted because this microorganism is frequently present in water and is extremely dangerous for human beings if it is found in aerosols [15-17]. The evaluation of the results highlights an effective diminution of the microbial load after 20 minutes of ozone treatment. We recorded some marked reductions, mainly regarding *S. aureus*, in agreement with a study by Cesar [18], and *S. faecalis*. *L. pneumophila* also showed a marked diminution. Ozone had a lower effect on *P. aeruginosa* and, particularly, on *E. coli*, although this latter microorganism is considered one of the bacteria most sensitive to ozone [18]. It is possible that, in this experimental condition, the concentration of the gas did not reach a sufficient level for the total elimination of *E. coli*, in accordance with a recent study conducted by the group of Heß, which reported that resistance to ozone inactivation probably depends on several factors [19].
In comparison with the other microorganisms, ozone exerted a small reduction on the mycetes load; we hypothesize that mycetes could be endowed with greater genetic resistance to this disinfectant.
The results on the microbial load present in the air in the room where the ozonization equipment was located showed limited significance, especially because the air which presented scant microbial contamination. Furthermore, another element that has to be considered is the large size of the room (85 $m^3$). As a consequence of the large volume of the room, ozone dispersion was elevated. The experiment should be repeated in a suitably smaller room in order to evaluate the positive impact of ozone on the microorganisms present in the air.
Furthermore, the surface of the mat which liberated the ozone was much smaller than that of a normal hydromassage bath tub. For these reasons, further studies should be performed, including the artificial contamination of the room; in the present study, this could not be done, since the room was used as a research laboratory.
Evaluation of the effect of the ozonized bath produced by Ozonomatic® on the microbial load in the aerosol yielded satisfactory results at a temperature of 36°C and regarding *E. coli, P. aeruginosa and S. aureus.* Concerning the *S. faecalis* load, a positive impact was also observed, though the reduction was less marked than in the other species analyzed.
The finding that at 22°C a moderate percentage reduction was found allows us to hypothesize that the microorganisms investigated could be endowed with greater resistance to this category of disinfectants.
The moderate effect exerted by ozone on the microbial load present in the air is strictly due to the mechanism of action of the ozone, which requires the presence of water.
The present study constitutes a preliminary investigation. Further research needs to be carried out in order to optimize sanitation parameters, including the evaluation of different times of ozone exposure, temperatures and volumes of the room where the ozonization equipment is located. Such factors can influence the effectiveness of antimicrobial ozone treatment.
In conclusion, ozone treatment is considered a safe and effective disinfectant tool for the decontamination of water and equipment [18] and even for food applications [20]; indeed, food safety is a top priority [21]. Ozone may therefore be regarded as a valid alternate means of disinfection.

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The authors declare no conflict of interest with Ozonomatic®.

## Authors' contributions

MM analyzed the data and prepared the manuscript. GF and RS carried out data collection and analysis. EM designed the study and performed data analysis and manuscript preparation. CT carried out the technical revision of the manuscript. All authors have critically read and revised the manuscript and approved the final version.

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ORIGINAL ARTICLE

# Effectiveness of hand hygiene education among a random sample of women from the community

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Keywords

Hand hygiene • Education • Awareness • Bacteria • Practice

Summary

**Objective**. *The effectiveness of hand hygiene education was investigated by studying the hand hygiene awareness and bacterial hand contamination among a random sample of 170 women in the community.*
**Methods**. *Questionnaire was used to assess the hand hygiene awareness score, followed by swabbing of the dominant hand. Bacterial identification was done by conventional biochemical tests.*
**Results**. *Better hand hygiene awareness score was significantly associated with age, scarce bacterial growth and absence of potential pathogen ($p < 0.05$). Out of the 170 hand samples, bacterial growth was noted in 155 (91.2%), which included 91 (53.5%) heavy growth, 53 (31.2%) moderate growth and 11 (6.47%) scanty growth. The presence of enteric bacteria was associated with long nails (49.4% vs 29.2%; p = 0.007; OR = 2.3; 95% CI: 1.25-4.44) while finger rings were associated with higher bacterial load ($p = 0.003$). Coliforms was significantly higher among women who had a lower hand hygiene awareness score, washed their hands at lower frequency (59.0% vs 32.8%; p = 0.003; OR = 2.9; 95% CI: 1.41-6.13) and used common soap as compared to antiseptic soaps (69.7% vs 30.3%, p = 0.000; OR = 4.11; 95% CI: 1.67-10.12).*
**Conclusions**. *Level of hand hygiene awareness among the participants was satisfactory but not the compliance of hand washing practice, especially among the elders.*

## Introduction

Lack of hand hygiene such as omitting hand washing after defeacation, changing baby nappies, and before handling food, could increase human contact with faecal matter [1]. The International Scientific Forum on Home hygiene has reported that hands could probably be the single most important route of transmission of large numbers of gastrointestinal, skin and respiratory tract infections. Bio-materials from the nose, eyes and skin during infections could contaminate the hands, which in turn could contaminate other fomites [2]. Hence, facilitating the spread of the infectious diseases. Proper hand washing could significantly reduce the transmission of pathogens from hands to food and other objects [3].
Hand hygiene promotion campaigns and practices has been reported to effectively reduce gastrointestinal infections by 31% and respiratory illnesses by 21% [2, 4]. In some countries, despite good quality of water, soaps and sanitary infrastructure are available, contagious infections associated with hygiene were found to be high in number. The reasons reported were lack of compliance and motivation to perform good hygiene practices [5-7]. This study was carried out in 2013 after a rigorous national hand hygiene awareness campaign by the Ministry of Health and Quality of life of Mauritius, using mass media to curb the transmission of influenza virus in the community. It would have been most appropriate to determine the effectiveness of the hand hygiene promotion campaigns by quantifying influenza virus using molecular methods from the hands of participants. However, it was not possible due to financial restraint. Therefore, we studied the effect of hand hygiene promotion campaign by measuring the hand hygiene awareness score and the presence of faecal bacteria as indicator from the hands of a random sample of female participants in the community.

## Methods

The study was conducted among a random sample of 170 female volunteers aged 12-60 years. Handicapped, elderly persons and individuals having occupations which could promote bacterial contaminations of hands, such as cleaners and healthcare workers were excluded. The participants who satisfied the inclusion criteria were asked to fill a self- administered questionnaire and a hand swab was taken by rolling a sterile swab moistened with peptone water, over the participant's palm, fingers and in-between the fingers of the dominant hand. Informed consent was obtained from the parent of the respondents who were less than 18 years of age. The study was approved by the Department of Health Sciences Research Ethics Committee of the University of Mauritius.

The questionnaire was designed to gather maximum information regarding the study such as age, socioeconomic status, occupations, frequency of hand-washing, whether they wash their hands with soap, length of nails and hand hygiene behaviours. The samples were immediately streaked on Blood agar, Mc Conkey agar and Salmonella Shigella agar. Bacterial growth and load was read after an incubation period of 24 hours at 37°C. The bacteria were identified by conventional gram staining, morphological and biochemical properties. Bacterial load was read as mean number of colony forming units (CFU). The presence of less than 20 CFU was read as scarce growth, 21-50 CFU as moderate growth and more than 50 CFU as heavy growth. Hand hygiene awareness score was based on questions such as whether hand washing was important, should hands be washed before handling food and if hand washing could prevent transmission of communicable diseases. The participant could score a minimum of 0 and a maximum score of 4. Data analysis was done using the statistical software SPSS v.16.0 (SPSS Inc, Chicago, IL, USA) and the level of significance was read as $p < 0.05$ for all analyses. Pearson correlation was used to determine relationship between the quantitative variables such as age of participant, length of nails, hand hygiene awareness score and microbial load. Pearson's chi-squared was used to determine any significant difference between hand hygiene behaviours and microbial load.

## Results

Out of the 170 women, 65.3% were aged 12-35 years and 34.7% between 36-60 years. The effect of age and other factors which could affect hand hygiene have been detailed in Table I. Bacterial growth was noted in 155 (91.2%) of the hand samples, with 91 (53.5%) heavy growth, 53 (31.2%) moderate growth and 11 (6.47%) scanty growth. The most common bacterium isolated was *Coagulase negative Staphylococcus* (45%), followed by *Streptococcus* spp. (37%), *Klebsiella* spp. (8%), *E. coli* (6%), *Bacillus* spp. (3%) and *Micrococcus* spp. (3%). A total of 100 (58.8%) of the respondents had a hand hygiene awareness score of four, 31 (18.2%) a score of three, 34 (20%) a score of two and 5 (2.9%) had a score of one. A higher hand hygiene awareness score was noted among the younger group ($p = 0.01$). Furthermore, scarce bacterial growth and absence of pathogenic bacteria were significantly associated with high hand hygiene awareness score.

The participants who reported to wash their hands more frequently, > 6 times/day, had higher hand hygiene awareness score ($p = 0.001$), scarce bacterial growth ($p = 0.004$) and coliforms (*E. coli* and *Klebsiella* spp.) were not detected from their hands. It was also noted that participants who were wearing rings at the time of sample collection, had heavier bacterial load ($p = 0.003$). Women who reported hand washing as a very important component of hand hygiene, were more likely to use antiseptic soap as compared to those who reported hand washing as less important (42.1% vs 6.1%; $p = 0.000$; OR = 2.55: 95% CI: 1.44-4.50).

The presence of coliforms was noted at higher prevalence among the women who had a lower hand hygiene awareness score ($p = 0.002$), washed their hands at lower frequency (59.0% vs 32.8%; $p = 0.003$; OR = 2.9; 95% CI: 1.41-6.13), used common soap as compared to antiseptic soaps (69.7% vs 30.3%, $p = 0.000$: OR = 4.11; 95% CI: 1.67-10.12) and had long nails (49.4% vs 29.2%; $p = 0.007$; OR = 2.3; 95% CI: 1.25-4.44). The younger group of participants had lower prevalence of coliforms, although the difference was not statistically significant (35.1% vs 45.8%; $p = 0.17$). Furthermore, coliforms were found from samples which had heavy bacterial load ($p = 0.000$).

## Discussion

Our results showed that hand hygiene awareness had a very important role among the participants. The younger participants were more knowledgeable on the matter and had lower prevalence of coliforms. It could be that the younger participants watched television or listened to the radio for longer period of time and therefore, they were more exposed to the hand hygiene campaigns than

**Tab. I.** Effect of age and other factors affecting hand hygiene.

| Factors affecting hand hygiene | 12-35 years (n = 111) % | 36-60 years (n = 59) % | p value | OR | 95% CI |
|---|---|---|---|---|---|
| Type of soap used | | | | | |
| Normal | 90.1 | 78.0 | 0.03 | 2.57 | 1.07-6.17 |
| Antiseptic | 9.9 | 22.0 | | | |
| Wear rings | 76.6 | 79.7 | 0.65 | 0.83 | 0.39-1.80 |
| Presence of long nails | 47.7 | 47.5 | 0.97 | 1.01 | 0.54-1.90 |
| Awareness of hand washing importance | 76.6 | 79.7 | 0.65 | 0.84 | 0.39-1.80 |
| Influence of hand washing campaign | 87.4 | 93.2 | 0.24 | 0.50 | 0.16-1.61 |
| Wash hands before handling foods | 71.2 | 88.1 | 0.01 | 2.43 | 1.14-5.17 |
| Hand type | | | | | |
| Normal | 78.4 | 64.4 | 0.04 | 2.00 | 1.01-4.03 |
| Sweaty | 21.6 | 35.6 | | | |
| Hand washing frequency | | | | | |
| Times | 23.4 | 22.0 | | | |
| > 6 times | 76.6 | 78.0 | 0.84 | 1.08 | 0.51-2.31 |

the elderly group. It has been previously reported that the lack of hygienic behaviours among adults (32-52 year) from developed countries could be because of their very busy lifestyle, false sense of health security due to high standard of water or sanitary facilities and incorrect belief that infectious diseases such as diarrhoea affected mostly children [5]. Furthermore, it has been suggested that positive outcome from hand hygiene promotion could be better achieved when people would practice hand hygiene not only more frequently but also at the right time [2].
Majority of the bacteria isolated were normal flora. *E. coli* and *Klebsiella* spp. have been increasingly reported to be associated with poor hygienic practices [8]. Previous studies have also reported that rings could contribute to hand contamination [9, 10]. In this study, the presence of long nails was associated with presence of coliforms. Various types of bacteria and parasites have also been isolated from the fingernail contents of food handlers [11].
The presence of coliforms from some samples indicated that the level of hand hygiene was below standard. In a similar manner, other highly infectious pathogenic microorganisms such as influenza viruses, rotaviruses and those responsible for foodborne illnesses could be present on the hands of people from community and transmitted. It should be noted that a high percentage of the participants had reported that their hand hygiene awareness and behaviours were positively improved after the hand hygiene campaigns. These findings indicated that the participants were not effectively translating their knowledge into practice. Previous studies have also reported similar behaviour [12]).

## Limitations

This study focused on bacterial contamination and not viruses, which are also very important in hygiene related infectious diseases. The hand hygiene behaviour reported by the participants might have been over-reported as some people might feel ashamed to disclose that they do not wash their hands whenever required.

## Conclusions

The participants had an overall acceptable hand hygiene score but compliance of hand washing practice was not always good. The population should be educated and reminded oftenly of the importance of hand hygiene in curbing incidence of infectious diseases, such as influenza, gastro-enteritis and conjunctivitis. It might be helpful to get NGO and university students on board to advocate for good basic hand hygiene practices in the community by adopting a door to door approach.

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## Authors' contribution

SBH supervised the study and UJ performed experimental work. SBH and UJ performed statistical analyses and wrote the manuscript. All authors read and approved the manuscript.

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ORIGINAL ARTICLE

# Association between type 2 diabetes mellitus and anthropometric measurements – a case control study in South India

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Keywords

Type 2 diabetes mellitus • Obesity • Body mass index • Waist circumference • Case control study • ROC curve

Summary

**Introduction**. *Obesity is a major risk factor for type 2 diabetes mellitus (T2DM). Clinical evidence indicates a stronger association of diabetes with central obesity than general obesity. The present study aimed to compare the association between type 2 diabetes mellitus and different anthropometric measurements and evaluate the usefulness of these measurements in clinical practice.*
**Methods**. *A case-control study was done among 102 individuals; of whom 51 cases included diagnosed T2DM (≥ 20 years age) patients attending the Medicine out-patient consultation of a tertiary care hospital and 51 controls who were screen negative for T2DM and recruited from the local community. Various anthropometric measurements were used according to standard World Health Organization (WHO) protocols. Data was entered and analyzed using Statistical Package for Social Sciences (SPSS) version 15.*
**Results**. *The proportion of cases with Body Mass Index (BMI) ≥ 25 kg/m$^2$ was 55% as compared to 22% of controls and this association was statistically significant ($p < 0.05$). The proportion of cases with high waist circumference cut-offs (WC) was 74.5% as compared to 45.1% healthy individuals and this association was also statistically significant ($p < 0.05$, OR = 3.56). A Receiver Operating Characteristic (ROC) curve for both gender revealed highest area under the curve for body mass index (area = 0.787). Body mass index had the best discriminatory power. Waist to hip ratio was not a sensitive marker especially for females.*
**Conclusions**. *A strong association between obesity indices and diabetes was identified. BMI and WC could be used in clinical practice for suggesting life style modifications.*

## Introduction

Diabetes is a major global health problem which the world is facing today. India is regarded as the diabetic capital of the world [1]. The emergence of type 2 diabetes mellitus (T2DM) in India, coinciding with the country's rapid economic development in the past several decades, is often characterized as a modern epidemic resulting directly from westernization [2]. The severity of the present situation in the Indian context can be judged from the alarming figures wherein, diabetes was directly responsible for 109,000 deaths, 1,157 years of life lost and 2,263 disability adjusted life years, in the year 2004 [3]. One of the major risk factor for T2DM is obesity. Clinical evidence indicates a stronger association of diabetes with central obesity than general obesity [4]. There are 380 million people in the world expected to have diabetes by 2025 [5]. In spite of a relatively lower rate of obesity as defined by Body Mass Index (BMI) cut points, South Asians tend to have larger waist measurements and waist-to-hip ratios (WHR), indicating a greater degree of central body obesity [6]. This is associated with a characteristic metabolic profile with higher insulin levels, a greater degree of insulin resistance, and a higher prevalence of diabetes [6]. Over the next 10 years in India deaths from chronic disease will increase by 18% - most markedly, deaths from diabetes will increase by 35% [7]. Simple anthropometric measurements have been used as surrogate measurements of obesity and have more practical value in both clinical practice and for large-scale epidemiological studies [8]. BMI is a simple method which is used to calculate the prevalence of overweight and obesity in the population. Waist circumference (WC) is the best measure of both intra-abdominal fat mass and total fat [8]. But BMI can be misleading, such as in individuals with a high proportion of lean muscle mass [9]. WC, a more accurate measure of the distribution of body fat, has been shown to be more strongly associated with morbidity and mortality [9]. Recently, the waist-to-stature ratio (WSR) has been proposed as a better screening tool than WC and BMI for adult metabolic risk factors [10]. The present study is aimed to compare the association between T2DM and different anthropometric measurements and evaluate the practicability and usefulness of these measurements in clinical practice and public health.

## Methods

This was a case control study comprising of 51 cases prospectively recruited from the hospital and 51 controls also recruited prospectively from the local community (Case control in the ratio of 1:1). Institutional ethical committee clearance was obtained (IEC 123/2014) before the initiation of the study. Written informed consent was obtained from all the study subjects. Information pertaining to socio-demographic characteristics and anthropometric measurements was collected by personal interviews using a pre-designed questionnaire.

### Cases: Patients with type 2 diabetes mellitus - Hospital

Cases were recruited from a tertiary care referral hospital in South India. Cases were the patients diagnosed with T2DM attending the tertiary care referral hospital. Inclusion criteria for cases was, age ≥ 20 years of both gender diagnosed with T2DM at least since two years, willing to participate and attending the Out Patient Department (OPD) clinic of Department of Medicine at the tertiary care referral hospital. Patients of T2DM having severe co-morbidities like stroke, chronic renal diseases and chronic lung diseases at the time of recruitment into the study; referred patients to the medicine OPD who came to the hospital due to other illness and pregnant females were excluded from the study. Cases were interviewed in the hospital and additional details about investigations, complications, etc. were obtained from OPD patient records for cases.

### Controls: Screen negative for diabetes - Community

Controls were the individuals not having T2DM, selected from the community in the field practice area of Department of Community Medicine. Controls were defined as individuals' ≥ 20 years of both gender, willing to participate and who were not suffering from type 2 diabetes mellitus. Diabetes was ruled out by screening the participants at the time of enrolment into the study by random blood glucose (RBS) estimation using a glucometer (Accu-Chek Active Blood Glucose Monitoring System). Subjects with RBS < 7.8 mmol/l [11] were eligible to be included as controls. Controls were approached by house to house survey in the field practice area of Department of Community Medicine, Kasturba Medical College, Manipal, which is operational for the last 50 years, and covers a population of 50,000.

Blood pressure was measured for all subjects in the study using a standardized mercury sphygmomanometer in the right arm in sitting posture. If the recorded blood pressure is ≥ 140/90 mm Hg (18.6/11.9 KPa), repeat blood pressure reading was taken after five minutes.

### Statistical analysis

Data was entered and analysed using Statistical Package for Social Sciences (SPSS) version 15 (SPSS Inc, Chicago, IL, USA) for Windows. Sample size was calculated by anticipating standard deviation of 0.09 and difference of 0.05 in WHR to be significant for power of 80% and 95% confidence interval, 51 cases and 51 controls (1:1 ratio) were recruited, group matched for gender.

All the variables were measured according to World Health Organization (WHO) guidelines and quality control was maintained during collection of data [12]. All the measurements were taken over light clothing. Weight was measured by mechanical weighing scale in kilograms to the nearest 0.5 kg, without footwear with the scale being placed on a firm flat surface. Height was measured by a measuring tape against a flat vertical surface and recorded in centimetres, to the nearest 0.1 cm. Waist circumference was measured by a measuring tape and recorded in centimetres, to the nearest 0.1 cm, at the mid-point between coastal margin and iliac crest. Hip circumference was measured by a measuring tape and recorded in centimetres, to the nearest 0.1 cm, at the level of maximum circumference of the ischial tuberosity of the participant.

The following ratios were calculated:

- WHR: waist circumference (cm)/hip circumference (cm);
- WSR: waist circumference (cm)/Height (cm);
- BMI: weight (kg)/height ($m^2$).

### Criteria for defining obesity

- BMI ≥ 25 kg/$m^2$ - overweight and BMI ≥ 30 kg/$m^2$ - obese [13].
- WSR ≥ 0.90 for males and ≥ 0.85 for females (truncal obesity) [14].
- WC > 90 cm in males and > 80 cm in females (central/abdominal obesity) [14].
- WSR > 0.5 [10].
- Blood pressure was classified according to Joint National Committee VII (JNC VII) criteria [15].

## Results

The study included 51 cases and 51 controls. Socio-demographic details of cases and controls were similar as depicted in Table I. The cases and controls were group matched by gender. Nearly 70% of cases belonged to age group of 40-59 years, while controls constituted 45% in the same age bracket. Hinduism being the predominant faith followed in the entire country is reflected in the study area too. Nearly one third of the population (37.3%) was illiterate among the cases, while control population in the community was more literate (86%).

The distribution of various occupation categories among cases and controls was similar. Over half of the study population belonged to the middle socio-economic category as per the modified Udai-Parikh scale used for socio-economic status assessment. The scale uses a scoring pattern based on household possessions, education and occupation of the eldest member of the family.

Among 75% of the subjects, the duration of diabetes was less than ten years. Among the cases, 63% were being treated only by oral hypoglycaemic agents while 31%

**Tab. I.** Socio-demographic characteristics of the study population.

| Demographic characteristics | Cases (n = 51) n (%) | Controls (n = 51) n (%) |
|---|---|---|
| Age group (years) | | |
| 20-39 | 1 (2) | 19 (37.2) |
| 40-59 | 35 (68.6) | 23 (45.1) |
| ≥ 60 | 15 (29.4) | 9 (17.6) |
| Gender | | |
| Male | 25 (49) | 26 (51) |
| Female | 26 (51) | 25 (49) |
| Religion | | |
| Hindu | 48 (94.1) | 50 (98.0) |
| Sikh | 0 (0) | 1 (2) |
| Christian | 3 (5.9) | 0 (0) |
| Education | | |
| Graduate and above | 6 (11.8) | 1 (2) |
| Elementary (1st to 12th Class) | 26 (50.9) | 43 (84.3) |
| Illiterate | 19 (37.3) | 7 (13.7) |
| Occupation | | |
| Professional/White collared | 1 (2) | 1 (2) |
| Business | 2 (3.9) | 8 (15.7) |
| Skilled | 13 (25.5) | 14 (27.5) |
| Semiskilled | 2 (3.9) | 1 (2) |
| Coolie/Unskilled | 2 (3.9) | 1 (2) |
| Not currently employed | 31 (60.8) | 26 (51) |
| Marital Status | | |
| Married | 51 (100) | 48 (94.1) |
| Unmarried | 0 (0) | 3 (5.9) |
| Socio-economic status | | |
| Low | 16 (31.4) | 12 (23.5) |
| Middle | 30 (58.8) | 39 (76.5) |
| High | 5 (9.8) | 0 (0) |

were both on insulin and oral hypoglycaemic agents. Hypertension (37%) was the most common co-morbidity among the cases as documented in the records. None of the diabetic subjects had their blood glucose levels within the suggested normal limits, as per the tests-fasting blood sugar (FBS), post prandial blood sugar (PPBS), random blood sugar (RBS) and glycated haemoglobin (HbA1c) mentioned in the patient records.

According to BMI categories (Tab. II) the proportion of cases with BMI ≥ 25 kg/m$^2$ among cases was 55% as compared to controls among whom only 21.6% individuals had BMI ≥ 25 kg/m$^2$ and this association was found to be statistically significant, with the odds being highest when BMI was ≥ 30 ($p < 0.05$).

The proportion of cases with more than normal waist circumference (Tab. II) was 74.5% as compared to 45.1% healthy individuals and this association was also found to be statistically significant ($p < 0.05$). Statistically significant association was also noted with WSR, but not with waist hip ratio (Tab. III).

BMI and WC were identified to have good sensitivity and specificity irrespective of gender (Tabs. IV, V).

The area under the curve for BMI, WC and WSR was 0.787, 0.734 and 0.737 respectively (Fig. 1) as per the Receiver Operator Characteristics (ROC) curve analysis for both gender. Among males (Fig. 2) all the anthropometric measures were found to have similar sensitivity, while among the females (Fig. 3) BMI was found to be the most sensitive marker fo rT2DM (Area = 0.818). WC and WSR were also good in assessing the risk of diabe-

**Tab. II.** Association between anthropometric variables among cases and controls.

| Anthropometric variables | Cases n (%) | Controls n (%) | Chi-square value | p value | Odds ratio | 95%CI |
|---|---|---|---|---|---|---|
| BMI category (Kg/m$^2$) | | | | | | |
| Underweight (< 18.5) | 3 (5.9) | 14 (27.5) | | | 0.27 | 0.07-1.10 |
| Normal (18.5-24.99) | 20 (39.2) | 26 (51) | 16.65 | 0.001 | 1.00 | |
| Overweight (≥ 25) | 14 (27.5) | 8 (15.7) | | | 2.27 | 0.79-6.47 |
| Obese (≥ 30) | 14 (27.5) | 3 (5.9) | | | 6.06 | 1.53-24.03 |
| WC | | | 9.18 | 0.002 | | |
| Normal | 13 (25.5) | 28 (54.9) | | | 1.00 | |
| Abnormal (M ≥ 90 cm) (F ≥ 80 cm) | 38 (74.5) | 23 (45.1) | | | 3.56 | 1.54 -8.22 |
| WHR | | | | | | |
| Normal | 9 (17.6) | 10 (19.6) | 0.06 | 0.799 | 1.00 | |
| Abnormal (M ≥ 0.90) (F ≥ 0.85) | 42 (82.4) | 41 (80.4) | | | 1.14 | 0.42-3.08 |
| WSR | | | | | | |
| Normal | 6 (11.8) | 18 (35.3) | 7.84 | 0.005 | 1.00 | |
| Abnormal (> 0.5) | 45 (88.2) | 33 (64.7) | | | 4.09 | 1.46-11.42 |

**Tab. III.** Cut-off values, sensitivity and specificity for different anthropometric measures.

| Anthropometric measure | Cut-off value | Sensitivity | Specificity |
|---|---|---|---|
| BMI | 21.85 | 82% | 65% |
| WC | 89.75 | 70% | 65% |
| WHR | 0.94 | 60% | 53% |
| WSR | 0.54 | 74% | 57% |

**Tab. IV.** Cut-off values, sensitivity and specificity for different anthropometric measures among males

| Anthropometric measure | Cut-off value | Sensitivity | Specificity |
|---|---|---|---|
| BMI | 22.07 | 76% | 66% |
| WC | 91.25 | 76% | 74% |
| WHR | 0.95 | 72% | 54% |
| WSR | 0.54 | 76% | 62% |

**Tab. V.** Cut-off values, sensitivity and specificity for different anthropometric measures among females.

| Anthropometric measure | Cut-off value | Sensitivity | Specificity |
|---|---|---|---|
| BMI | 22.28 | 80% | 68% |
| WC | 83.5 | 73% | 60% |
| WHR | 0.94 | 46% | 48% |
| WSR | 0.54 | 73% | 56% |

tes. WHR was not found to be suitable marker for T2DM among females.

## Discussion

The present study identified WC, WSR and BMI to be associated with T2DM than WHR. The odds of a diabetic individual having high waist circumference was 3.56 times more as compared to a non-diabetic individual.
Different anthropometric cut-off values for various ethnic groups and populations, always makes comparisons difficult and limits generalizability. In most of the studies BMI performed poorly as an anthropometric predictor for T2DM which was in contrast to the present study where BMI was found to be the sensitive marker for diabetes and especially among females. In the Uppsala study [16] they concluded that overweight (BMI 25-30 kg/m$^2$) or obese men (BMI 30 kg/m$^2$) without metabolic syndrome were at increased risk for diabetes which were comparable with our results. In the present study, WHR was not a sensitive marker for T2DM. But there are contrasting views on this anthropometric measure in literature [1, 17].
WC was found to be a significant predictor of T2DM in a systematic review [18] and a prospective cohort study [19] which was concurring with the present study findings. Results from a systematic review [10] and a multi ethnic cohort study [20] identified WSR to be a

**Fig. 1.** ROC curve for both gender.

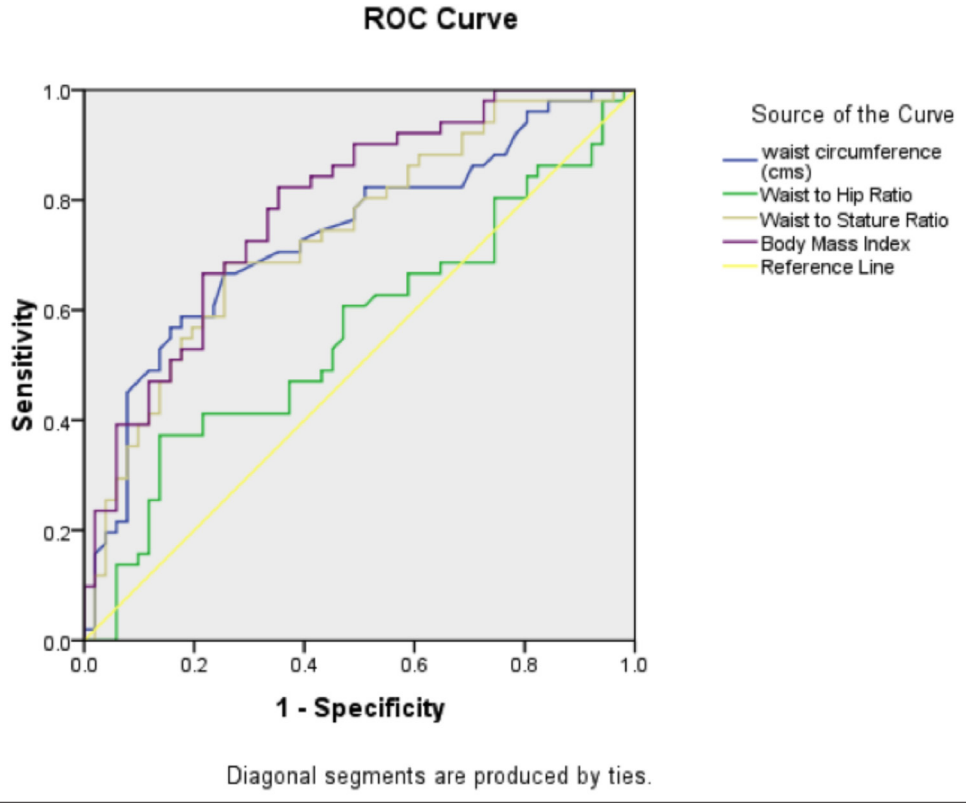


Fig. 2. ROC curve for males.

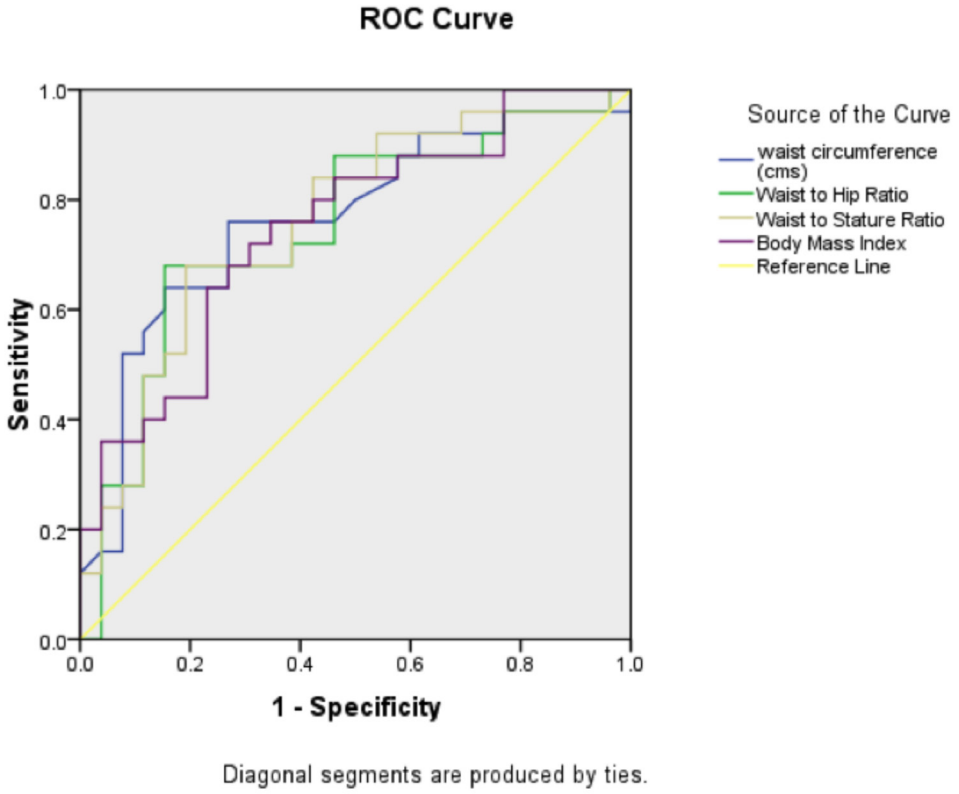
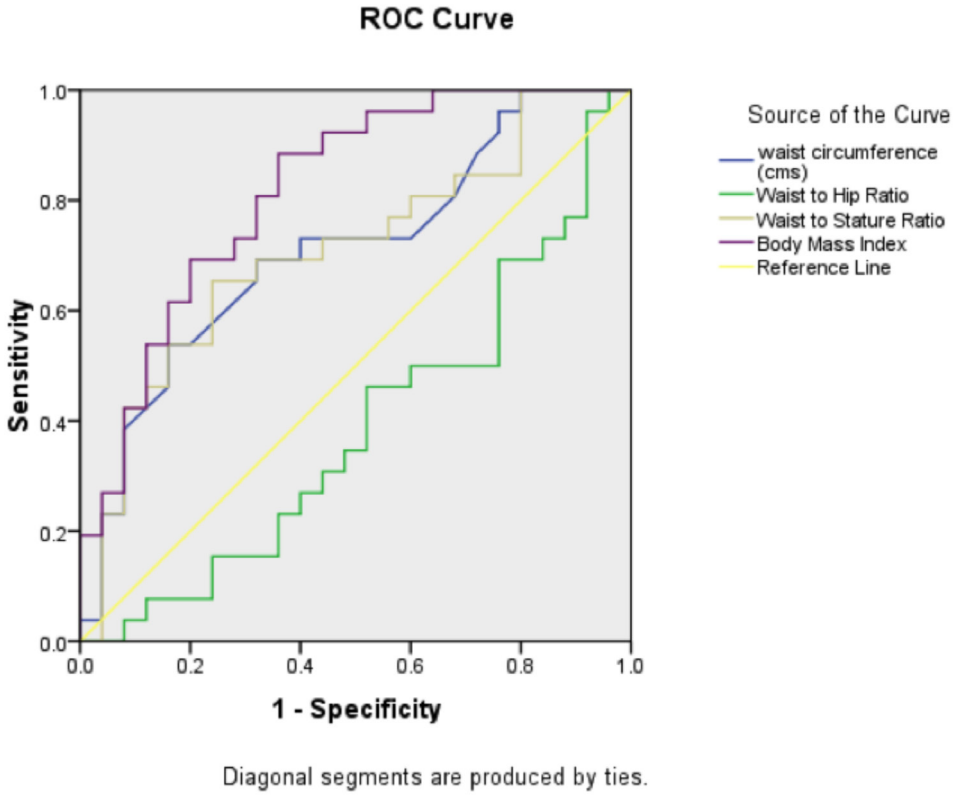



Fig. 3. ROC curve for females.



more useful clinical screening tool similar to the present study.

The present study was an effort to identify the discriminatory power of various anthropometric measures and its association with T2DM, using hospital cases and community controls. Despite the small sample size, undisputedly waist circumference may be endorsed as the single most convenient, feasible measure that could be used across communities for its significant association with T2DM.

Generalizability of the results is a limitation of the study because of the smaller sample size and due to disparities in various cut-offs used to define obesity in the available literature. Assessment of glycaemic control was intended to be assessed using investigation reports available from patient's files, but could not be done satisfactorily due to discrepancies in data records. A cohort study with a larger sample size is recommended to determine the optimal cut-off points for the various anthropometric measurements specific for the Indian population.

## Conclusions

Among the various anthropometric measurements, BMI was found to have the best discriminatory power. WC and WSR were also found to be sensitive markers. WHR was not a sensitive marker especially for females. So, waist circumference as a single measure could be advocated due to simplicity of measurement and usage either in hospital or community settings.

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## Author contributions

AA, CRR contributed equally to this work; AA, CRR designed the research, analysed the data and drafted the paper. AA, CRR, DSH, KRN have provided substantial contributions to the acquisition and analysis of data for the work; CRR revised the manuscript critically for important intellectual content; reviewing the final version to be published and agree to be accountable for all aspects of the work.

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ORIGINAL ARTICLE

# Difference in quality of life and associated factors among the elderly in rural Vietnam

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## Keywords

Quality of life • Elderly • Gender differences • Related factors • Vietnam

## Summary

**Background**. *In Vietnam today, many generations remain living together in a family. With escalating urbanization and population aging, mental health disorders and the quality of life (QoL) among the elderly are gradually presenting themselves as of great concern. The objective of this study was to examine gender differences in QoL and some associated factors among the elderly in rural Vietnam using the QoL scale of WHO (WHOQOL-BREF).*
**Methods**. *A cross-sectional study using quantitative methods.*
**Results and Conclusions**. *The proportion of the elderly men having higher level of QoL in physical health, psychological health and environment was higher than that of their women counterparts. Reversely, of those having medium and lower QoL, females made up a larger proportion than males. The overall QoL score in elderly men (75.32) was higher than that of women (72.32) and the same pattern was witnessed in all four domains of QoL. While higher QoL in elderly men was significantly correlated with 5 factors, aged ≥ 80 years, following Buddhism and Christianity, having better connection and without illness in the past 6 months, these among female counterparts are aged ≥ 80 years, completing secondary level or above, having medium and high socioeconomic status and without illness in the last 6 months.*

## Introduction

Recently, Vietnam has witnessed an increasing aged population. According to the United Nations Population Fund (UNFPA), the elderly population in Vietnam, which increased from 3.71 million (1979) to 7.72 million (2009), is anticipated to reach 12 million people in 2020 [1]. It is concerned that this rapid growth may present great challenges to the provision of social services, including health care for older people. It is also worth noting the sensitive nature of the elderly's mental life. With the passing of time, Vietnamese seniors are increasingly prone to negative feelings, regarding themselves as onerous burdens to their descendants. Some people, after retirement, tend to feel lost and shunted sideways by the society. For this reason, the elderly are among susceptible age groups that should be attended to not only physically, but also emotionally. Considering the profound influence of quality of life (QoL) upon their well-being, the research on QoL among the elderly in a continuously changing context will provide significant inputs for designing and implementing appropriate policies and programs with regard to the enhancement of their QoL. The change of the quantity and structure of the elderly leads to changes of disease pattern, including changes of quality of life. Biological differences between elderly men and elderly women also have gender differences in quality of life.

Despite numerous prior studies on QoL in general among the elderly, few interesting studies on gender differences have been found. For instance, a study among the elderly in rural Thailand [2] indicated that more elderly females reported lowest and medium QoL than men, while more men reported highest QoL. Another study among the elderly living in rural and urban India showed while there was no gender difference in average score of QoL in three domains, physical health, psychological health and social relationships, a statistically significant difference in environmental QoL was detected with the average score of elderly men higher than that of women [3]. QoL among the elderly in general is subject to many factors. According to Mudey [3], a number of factors were associated with low QoL among the Indian elderly, separation from a spouse, poor socioeconomic status, lack of regular exercise, sleeping or hearing difficulty, suffering one kind of illness and history of a fall within the last 6 months. In Nigeria, traditional lifestyle, educational level, socioeconomic status, gender and marital stability were closely related to QoL of many senior citizens [4]. In addition, environment is also among the major determinants of high QoL. Living in a crowded environ-

ment had a negative impact on individuals' health status and QoL in the future [5, 6]. The role of other factors, recreational activities, job satisfaction and life security [5] was also important. A review by Djernes revealed that lack or loss of closeness and intimacy in social contacts were likely to predict depression and low QoL among the elderly [7]. In a study comparing QoL among senior citizens in Vietnam and Bangladesh, factors such as socioeconomic status, living environment, social relations, religion and beliefs are intimately associated with health and QoL [8]. Still, there is insufficient evidence available regarding gender differences in QoL of the elderly [9, 10] and little has been known about factors associated with QoL among each group of gender, male and female elderly.

Although QoL in Vietnam remained relatively lower compared to other countries in the world (116/182 countries), it ranked 4th in Southeast Asia and Association of South-East Asian Nations (ASEAN) after Laos, Cambodia and Myanmar [11]. However, the QoL among the elderly up to date remains in question, although there are some prior data on this regard. For instance, in 2009 when evaluating just the instrument for measuring QoL in Vietnam, Huong et al. revealed a higher average score of QoL in elderly men than elderly women [12]. Another study of Xoan in 2012 showed the similar pattern with more women reporting lower and medium QoL versus more men reporting higher QoL [13]. A study by Hoi et al. on QoL of 600 seniors in rural district of Hanoi, Vietnam using European scale of QoL (EQ-5D Index) also revealed a higher average score of QoL in elderly men than elderly women [14]. Although some of the prior studies as mentioned suggested differences in QoL between elderly men and women, these differences along with QoL related factors have not been elaborated. Moreover, inconsistency in using the WHO scale of QoL might impede the comparison and evaluation to a certain degree. A study about QoL among people aged 50+ years by Ninh Thi Ha et al., WHOQOL-BREF is a reliable instrument to measure QoL among hypertensive patients. The results revealed low QoL in psychological domain and inequality in QoL across socio-demographic characteristics [15].

Given limitations of previous research on gender differences in QoL, the purpose of this study was to examine gender differences in QoL and factors associated with it by gender in rural district of Thai Binh province, Vietnam. A conceptual model for this research was based on a model by Kamp et al. (Fig. 1). This theoretical framework consists of six factors influencing health, security, physical environment, personal development, resources and community development as contributors to QoL [16]. We used this model of Kamp et al. in our study because it is relatively comprehensive and applicable to many research subjects including older people. As we used in the elderly population, it is necessary to adapt it, especially in rural setting of developing countries. The factors used in adapting the Kamp model were identified based on the previous research in people living in rural areas of some European countries [16]. In the current context of Vietnam, we focused on a number of factors, health (mental health and physical health); physical environment; natural resources; goods and services; community development; and personal development.

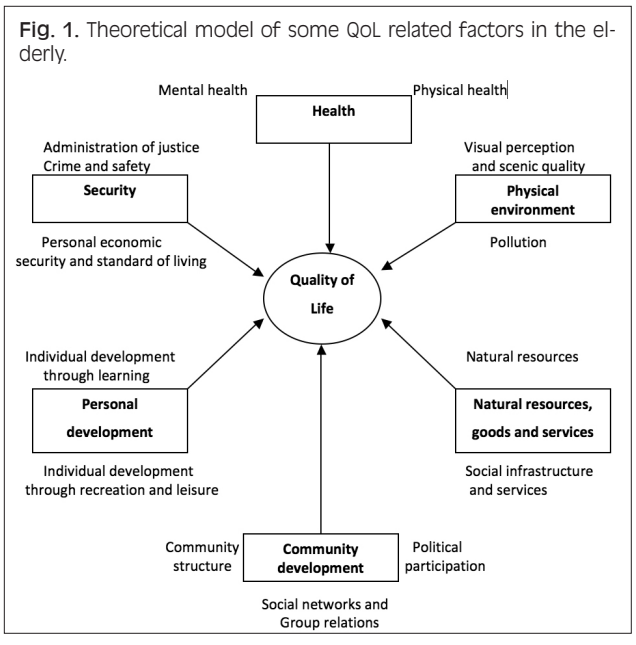


**Fig. 1.** Theoretical model of some QoL related factors in the elderly.

## Methods

### Design

This is a cross-sectional study adopting quantitative method with face-to-face interviews using WHO instruments to measure QoL, WHOQOL-BREF [17].

### Sites and sampling

The study was carried out in three communes of Kien Xuong district in Thai Binh province. Thai Binh is the granary of Northern Vietnam, one of the strategic provinces in the national target program on new rural construction which has achieved encouraging successes in improving living standards in rural areas. Kien Xuong is an agricultural district in Thai Binh province. With a large area of 200 km$^2$, it is located in the north west of Thai Binh city, consisting of one town and 36 communes. Its population was 274,318 people in 2012, including 34,898 senior citizens [18]. We chose these communes for the study because they could represent a typical rural community in Vietnam such as income sources mainly from agriculture, an increasing number of the elderly people, and quite far from urban setting; they also have received limited support and interventions to improve QoL of the elderly over the past decades [19].

Participants were screened if they were elderly aged 60 years or more, living in the selected area for at least 5 years, being available at the time of the survey and willing to participate voluntarily in the survey. The sample size was generated based on the sample formula to test the difference between the proportion of elderly males and females in QoL:

$$n = Z^2_{(a,b)} \frac{p_1(1-p1) + p_2(1-p2)}{(P_1-P_2)^2}$$

Where: $p_1$ is the percentages of higher QoL among elderly men and women, respectively. In a previous study in Myanmar in 2010, $p_1$ was 0.218 and $p_2$ was 0.135 [20]; $(P_1-P_2)$ is the desired relative precision higher QoL between elderly men and elderly women. The desired precision in our study was set at 0.30; a is the significant level and the probability of making type I error (reject the null hypothesis $H_0$ when it is true). Take a = 0.05 with the 95% confidence interval, and b is the probability of making type II error (accept $H_0$ when it is false) take b = 0.1. Then $(Z_{(a,b)})^2 = 10.8$

Using these above values, a sample size of 402 elderly subjects were calculated.

Applying systematic random sampling technique the study subjects were chosen in two phases: phase 1, sample 3 communes in Kien Xuong district from Thai Binh province, and phase 2, select the number of participants in each commune using by systematic random sampling. From the data provided by People's Committee in three communes: make a list of the elderly, then identify k-interval in each commune (average of k-interval = 4).

## Measurement

### Dependent Variable

***QoL:*** Given the prior literature, there have been quite numerous QoL measures widely studied. Of the QoL measures, the Quality of life Instruments developed by WHO (WHOQOL-BREF) has been one of the most widely used tools in QoL research as it is able to assess individual perceptions in the context or their culture, personal goals, standards and concerns [9, 21] as well as has been widely field-tested and validated [22, 23]. We adopted his tool as it was developed for international cross-culturally comparable QoL evaluation and have been widely field-tested [22, 23].

In our current study, when examined for internal consistency, with 26 items, the WHOQOL-BREF instrument was of relatively high consistency [Cronbach's alpha (a) = 0.89, and each item presents one facet of QoL and two "benchmark" items for an individual's overall QoL and general health]. The facets were defined as aspects of life considered to have contribution to a person's QoL. QoL comprised four main domains-physical health (7 items, a = 0.73), psychological health (6 items, a = 0.76), social relationship (3 items, a = 0.63) and environment (8 items, a = 0.66). These facets were scored on a Likert scale of 1 to 5 with 1 = very poor, 2 = poor, 3 = neither poor or good, 4 = good and 5 = very good; and 1 = very satisfied, 2 = dissatisfied, 3 = neither dissatisfied or satisfied, 4 = satisfied and 5 = very satisfied; 1 = not at all, 2 = a little, 3 = a moderate amount, 4 = very much and 5 = extremely; or 1 = never, 2 = seldom, 3 = quite often, 4 = very often and 5 = always.

If the use of scores obtained in the WHOQOL-BREF domains is a goal to be pursued in this research, additional clarification of factors that influence the responses in distinct areas becomes a key objective to be explained. Therefore, categorizations of the physical, psychological, social relationships and environmental factors QoL score is necessary. The scores for the physical health assessment were divided into 3 levels: 7-16 points, low level; 17-26 points, middle level; and 27-35 points, high level QoL. The scores of the psychological health assessment were divided into 3 levels: 6-14 points, low level; 15-22 points, middle level; and 23-30 points, high level mental QoL. The scores for the social relationships assessment were divided into 3 levels: 3-7 points, low level; 8-11 points, middle level; and 12-15 points, high level of quality of social relationships. The scores of the environmental factors were divided into 3 levels: 8-18 points, low level; 19-29 points, middle level; and 30-40 points, high level environmental factors. The overall interpretation of the quality of life was divided into 3 levels: 26-60 points, low level; 61-95 points, middle level; and 96-130 points, high level QoL [24].

### Independent Variables

***Demographics:*** demographical data were collected including age groups (60-79 and ≥ 80 years old), marital status (single, divorced, widowed, and married), religion (no religion, Christianity and Buddhism); educational level (illiteracy, not completing primary level, not completing secondary level, secondary level or above), living circumstance (living with spouses, children, friends, relatives or living alone).

***Social connection:*** the indicator of social connection was measured with seven yes/no items and is the sum of 7 variables of social engagement (retirement clubs, women's associations, veterans' associations, communist party cells, sport clubs and street population groups, religious groups). The number of organizations that an individual participated in correspondence to the number of point. As this scale was of relatively high reliability (Cronbach's $\alpha$ = 0.67) and of good construct validity (most of the factor loading coefficients or b's values > 0.30), we formed a composite of all items as a single common variable. In particular, a composite score was obtained by summing responses to items with higher composite scores indicating higher levels of social connection.

***Socioeconomic status:*** it was assessed with three levels of income (low, medium and high). The classification of socioeconomic status was based on the self-reported assessment of elderly themselves.

***Illness during the past 6 months:*** it was measured with yes/no item asking participant if she/he got ill over the past 6 months.

### Data collection

Prior to the survey, the field researchers were trained to become familiar with the key contents of research, survey methods, data collection, sampling methods, selecting study subjects, recording the responses of study subjects.

In the survey, participants were introduced about the objectives and main content so they can decide to participate voluntarily. Those who were not available at the interview time were set another appointment. Each in-

terview lasted about 30-45 minutes. The interviews took place within 2 months from June 2013 to August 2013. During the survey, the principal investigator was present in the field to supervise data collection.

### Statistical analysis

Data were cleaned by checking missing data before being entered into database using Epidata software. The data continued to be cleaned for outliers and illogical data and were converted into Stata version 12.0 file for analysis.

Each item of physical health, psychological, environment and social relationships domain was scored on a Likert scale of 1 to 5. The total score of each domain was then computed to form a scale of 0 to 100 with low scores reflecting poor QoL. A domain was treated as missing when over 20.0% of its items were missing.

Both descriptive and inferential statistics were used. Means and standard deviations (SD) were for continuous variables if data are normally distributed. Percentages were presented for nominal variables. As QoL scores were normally distributed, we applied parametric tests for continuous variables. To be specific, inferential tests included t-test to compare means between groups and chi-square test to compare the gender proportions in QoL. Linear regression was used to detect the fit model. To be specific, coefficient $R^2$, standardized b and model fit indicators were critically looked to detect factors associated with the level of the QoL among the elderly. To find a suitable model both bivariate and multiple linear regression analysis were employed. The final model was

**Tab. I.** General information of the elderly by gender.

| Characteristics (n = 402) | | Male ($n_1$ = 201) | Female ($n_2$ = 201) | General |
|---|---|---|---|---|
| | | Frequency (%) | Frequency (%) | Frequency (%) |
| Age groups | 60-69 | 100 (49.8) | 121 (60.2) | 221 (55.0) |
| | 70-79 | 70 (34.8) | 60 (29.9) | 130 (32.2) |
| | ≥ 80 | 31 (15.4) | 20 (10.0) | 51 (12.7) |
| Religion | None | 184 (91.5) | 170 (84.6) | 354 (88.1) |
| | Buddhism | 14 (7.0) | 28 (13.9) | 42 (10.4) |
| | Christianity | 3 (1.5) | 3 (1.5) | 6 (1.5) |
| Educational level | Illiteracy | 4 (2.0) | 13 (6.5) | 17 (4.2) |
| | Not completing primary level | 35 (17.4) | 57 (28.4) | 92 (22.9) |
| | Not completing secondary level | 49 (24.4) | 47 (23.4) | 96 (23.9) |
| | Secondary level | 75 (37.3) | 63 (31.3) | 138 (34.3) |
| | High school level | 27 (13.4) | 10 (5.0) | 37 (9.2) |
| | College or above | 11 (5.5) | 11 (5.5) | 22 (5.5) |
| Marital Status | Single | 13 (6.5) | 16 (8.0) | 29 (7.2) |
| | Divorced | 6 (3.0) | 12 (6.0) | 18 (4.5) |
| | Widowed | 26 (12.9) | 59 (29.4) | 85 (21.1) |
| | Married | 156 (77.6) | 114 (56.7) | 270 (67.2) |
| Caregiver | Yes | 193 (96.0) | 188 (93.5) | 381 (94.8) |
| | No | 8 (4.0) | 13 (6.5) | 21 (5.2) |
| Previous occupation | None | 1 (0.5) | 3 (1.5) | 4 (1.0) |
| | Farmer | 121 (60.2) | 140 (69.7) | 261 (64.9) |
| | Civil servant | 42 (20.9) | 23 (11.4) | 65 (16.2) |
| | Manual worker | 23 (11.4) | 23 (11.4) | 46 (11.4) |
| | Househusband/ housewife | 1 (0.5) | 6 (3.0) | 7 (1.7) |
| | Freelance | 8 (4.0) | 5 (2.5) | 13 (3.2) |
| | Others | 5 (2.5) | 1 (0.5) | 6 (1.5) |
| Living circumstance | Alone | 12 (6.0) | 20 (10.0) | 32 (8.0) |
| | With other people | 189 (94.0) | 181 (90.0) | 370 (92.0) |
| Social connection | Yes | 162 (80.6) | 163 (81.1) | 325 (80.8) |
| | No | 39 (19.4) | 38 (18.9) | 77 (19.2) |
| The main source of income | Wage/ Salary | 31 (15.4) | 29 (14.4) | 60 (14.9) |
| | Personal savings | 37 (18.4) | 26 (12.9) | 63 (15.7) |
| | Pension | 59 (29.4) | 34 (16.9) | 93 (23.1) |
| | Depending on spouse | 20 (10.0) | 28 (13.9) | 48 (11.9) |
| | Depending on descendants | 39 (19.4) | 66 (32.8) | 105 (26.1) |
| | Social welfare | 15 (7.5) | 18 (9.0) | 33 (8.2) |
| Socioeconomic status | Low | 40 (19.9) | 55 (27.4) | 95 (23.6) |
| | Average | 131 (65.2) | 133 (66.2) | 264 (65.7) |
| | High | 30 (14.9) | 13 (6.5) | 43 (10.7) |

selected through a process of performing stepwise linear regression, if $R^2$ was of great value and other indicators of model fit were met. Statistical significance was set at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

### Research ethics

The research was approved by the Scientific and Education Panel from the Institute for Preventive Medicine and Public Health, Hanoi Medical University. During the survey, participants were verbally informed about the study that their participation was voluntary, that they had the right to withdraw at any point, and that data would be confidentially managed. All the respondents was anonymous in the study.

## Results

### Key characteristics of the sample

In Table I, among 402 elderly people interviewed, the majority was in the age of 60-69 groups (49.8% males and 60.2% females) while those aged 80 accounted for a small proportion. Most of the elderly were nonreligious (88.1%), had completed secondary level (49.0%) and were married (67.2%). Most of the elderly were living with their families and being cared for. Previous occupations were mainly farmers. 26.1% of participants depended on their descendants, which was higher than the percentage of those having wages, salaries, and pensions and depending on their spouses. Overall, the majority of the elderly were of low and average socioeconomic status.

### Quality of life of the elderly

In Table II, QoL of the elderly men was higher than that of women ($p < 0.05$). In physical health, at high QoL, the percentage of males (6.5%) was higher than that of females (0.5%); at middle and low level, the percentage of females was higher than that of males ($p < 0.01$). As regards psychological health, at high level, the percentage of men was higher than that of women (12.4 and 2.0%),

**Tab. II.** The distribution of QoL in the elderly by gender.

| Dimensions of QoL | Male ($n_1$ = 201) | Female ($n_2$ = 201) | p-value (chi-square) |
|---|---|---|---|
| | Frequency (%) | Frequency (%) | |
| Physical health<br>Low<br>Middle<br>High | <br>18 (9.0)<br>170 (84.6)<br>13 (6.5) | <br>25 (12.4)<br>175 (87.1)<br>1 (0.5) | ** |
| Mental health<br>Low<br>Middle<br>High | <br>21 (10.4)<br>155 (77.1)<br>25 (12.4) | <br>22 (10.9)<br>175 (87.1)<br>4 (2.0) | *** |
| Social relationships<br>Low<br>Middle<br>High | <br>10 (5.0)<br>143 (71.1)<br>48 (23.9) | <br>10 (5.1)<br>158 (78.6)<br>33 (16.4) | |
| Environment<br>Low<br>Middle<br>High | <br>3 (1.5)<br>172 (85.6)<br>26 (12.9) | <br>12 (6.0)<br>176 (87.6)<br>13 (6.5) | ** |
| Overall<br>Low<br>Middle<br>High | <br>11 (5.5)<br>182 (90.5)<br>8 (4.0) | <br>20 (10.0)<br>180 (89.6)<br>1 (0.5) | * |

$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

**Tab. III.** The distribution of scores of QoL among the elderly by gender.

| Factor (n = 402) | Male | Female | p-value (t-test) |
|---|---|---|---|
| | X ± SD | X ± SD | |
| Physical health | 21.04 ± 3.60 | 20.04 ± 3.07 | ** |
| Psychological health | 18.84 ± 3.14 | 18.11 ± 2.84 | * |
| Social relationships | 10.26 ± 1.60 | 10.13 ± 1.53 | |
| Environment | 25.20 ± 3.53 | 24.03 ± 3.70 | ** |
| Overall | 75.32 ± 9.94 | 72.32 ± 9.21 | ** |

$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

at medium and low level, elderly women accounted for a higher percentage than elderly men ($p < 0.001$). However, in social domain, there was no gender difference found at all 3 levels. As for environment domain, at high level, the elderly men amounted to a higher percentage than women (12.9 and 6.5%) whereas the reverse was true at medium and low levels ($p < 0.01$).

According to Table III, the average scores of QoL in the elderly men (75.32) was overall higher than elderly women (72.32) ($p < 0.01$). In 3 out of 4 domains, the elderly men had higher average scores than their female counterparts ($p < 0.05$ and $p < 0.01$). However, no gender difference was found in the average score for social relationships.

### Factors associated with QoL among elderly men and elderly women

For women, factors related to better QoL included being at the age of 60-69, completing secondary level or above, previously working in the public sector, having pensions or descendants to rely on, having medium or high socioeconomic status, not widowed; having favorable social relationships and social engagement; having close social relationships, and experiencing no illness in the past 6 months. Among these factors, social relationships and social engagement and health status in the last 6 months have considerable association with QoL in both genders (with high value of standardized regression coefficient b and $p < 0.001$).

Table IV compares the correlation of a number of factors with QoL by gender. In both elderly men and women, those aged ≥ 80 and experienced illness in the past 6 months were more likely to have lower QoL than their younger counterparts ($|b| > 0.2$, $p < 0.05$). However, in elderly men, there were other factors associated with QoL, including Buddhism and Christianity, and having good social connection. For elderly women, completing secondary level or above, having medium and high socioeconomic status were the predictors of higher QoL.

## Discussion

### Gender differences in QoL between elderly men and elderly women

Our study showed that QoL of elderly men was generally higher than women and there were discernible gender differences in all four domains and thatmen displayed a higher percentage of men with high QoL than women and a higher percentage of women with low QoL than men. Our findings resemble some prior studies in both developed and developing countries. The study by Apidechkul in 2011 on elderly people in rural and suburban areas in Thailand, for instance, showed that the percentage of elderly women (2.9%) with low level of QoL was higher than men (0.0%) whereas at high level, the former was lower with 33.8% and 50.0%, respectively [2]. However, at average level, the percentage of elderly women was lower than that of elderly men [2]. Our results are also consistent with that of Huong's study in Hai Duong which indicated a higher percentage of men at high level of QoL with 3.4% compared to 2.7% of women [12]. Research conducted in Myanmar was also of the similar result with 21.8% and 13.5% respectively [20]. These similarities are possibly derived from the fact that our research was carried out in the rural setting which has the population with the same

**Tab. IV.** Some factors associated with QoL in the elderly by gender (*the results of multivariable analysis*).

| Independent variables | Male | Female |
|---|---|---|
| | Standardized regression coefficient b | Standardized regression coefficient b |
| Age<br>60-69<br>70-79<br>≥ 80 | <br><br>0.04<br>-0.2* | <br><br>-0.1<br>-0.3*** |
| Religion<br>None<br>Buddhism<br>Christianity | <br><br>0.2**<br>0.13* | <br><br>0.01<br>-0.1 |
| Educational background<br>Illiteracy<br>Not completing primary level<br>Not completing secondary level<br>Secondary level or above | <br><br>0.0<br>-0.04<br>0.1 | <br><br>-0.02<br>0.14<br>0.31** |
| Socioeconomic status<br>Low<br>Average<br>High | <br><br>0.1<br>0.1 | <br><br>0.17*<br>0.2** |
| Social connection | 0.3*** | 0.04 |
| Illness during the past 6 months | -0.2** | -0.23*** |
| Adjusted R2<br>Model fit indicator (F)<br>p | 0.523 (52.3%)<br>15.1<br>*** | 0.39 (39.0%)<br>6.22<br>*** |

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

cultural, economic and environmental characteristics as those in other studies and/or in other settings.

Regarding the distribution of average points of QoL between elderly men and women, gender differences were also found. In fact, the data showed that the elderly men had higher average score than their female counterparts. This result is similar to that of previous research on rural Vietnam with 239.3 for men and 228.7 for women in Huong's study and 230.2 and 220.9 [12] respectively in Xoan's study [13]. Research of Luong in Hai Duong also revealed a higher average score in men with 64.1 ± 10.4 compared to women with 61.0 ± 10.1 (25). Some reports by Nilsson on the elderly living in rural Bangladesh also gave the similar findings (2005) [8-10].

Looking specifically at 3 dimensions of QoL (physical health, psychological health and environment), it was found that elderly men also had higher average scores than elderly women. Our results were consistent with those of Nilsson's study on QoL of seniors in rural Bangladesh [9]. This can be explained by the cultural norms that male and female roles differ in the family and society that can affect QoL. Currently in many developing countries including Vietnam, women are expected to assume two responsibilities concurrently, work commitments and home chores. They not only have their own career to handle but also have to assume the role of main caregiver at home, which exert unavoidable pressure both on their professional and personal lives to a certain extent. In Vietnam, women sometimes suffer from domestic violence, especially in rural areas, which massively affect their mental life and QoL. According to the 2010 report of General Statistics Office of Vietnam and United Nations, more than half of Vietnamese women (58%) had ever fallen victim to at least one form of domestic violence (physically, sexually and mentally) triggered by their spouses [26]. While husbands and wives both make contribution to household income, women are actually dedicated to family more than their male partners considering their contributing time and effort. Yet, most major decisions in the family are normally made by men whereas women only sort out minor issues related to housework and caregiving. Our results also resemble those of the research of Mudey et al. on average scores of QoL among Indian elderly [3]. This resemblance is explainable as both Vietnam and India located in Southern East Asia share many similarities in socioeconomic status, cultural characteristics and living environment. Due to this high profile of gender differences in QoL among the elderly, it is crucial to understand what factors would affect determine QoL in each gender.

### Factors associated with QoL in elderly men and elderly women

According to the result of multivariate linear regression, it was identified that predictors of QoL differed between elderly women and men. Yet, in both sexes, those who belonged to the oldest age group (aged 80 and above) and experienced illness in the past 6 months had higher QoL than other elderly. The common tendency is that aging is very often accompanied by impaired health, increased vulnerability to ailments, and fewer opportunities for social engagement, which in turn lead to a decrease in QoL. Some other studies in the world also accentuated the influence of health on the QoL of the elderly [27]. In 2010, the research of Luong revealed the intricate relationship between age and QoL, the higher the age, the lower QoL [25]. Our results are also in concert with several studies in other countries such as of Jan (2005) and Rana (2009) in rural Bangladesh [9, 18]. In 2010, the research of Luong revealed the intricate relationship between age and QoL, the higher the age, the lower QoL [25].

For elderly women, however, their QoL was significantly associated with educational level and socioeconomic status. Those who graduated secondary level and above and having average socioeconomic status or above were more likely to have desirable QoL than those who had lower level of education and socioeconomic status. Naing's findings in Myanmar also indicated that those who had more favorable socioeconomic status had better QoL than others [20]. As identified in Nigeria [4], educational level, socioeconomic status, genders and marital stability had a certain impact on QoL of the elderly. Among elderly men, the QoL is associated to several factors including Buddhism and Christianity, socioeconomic status and social connection. Those who followed Buddhism and Christianity, had better socioeconomic status and better social connection were likely to have better QoL. Generally in developing countries, males have a tendency to enjoy more privileges than their female counterparts now that they have better constitution and have more opportunities to participate in political, cultural, social activities than females. Our findings which are relevant to this perception also tally with the findings of previous studies. Research of Luong on the elderly in Hai Duong, Nilsson on the elderly in rural Bangladesh and Bowling 2002 all confirmed the relationship between social connections and QoL of the elderly [10, 25, 27, 28].

Also, according to our study, social engagement including socializing and visting pagodas is important to QoL. This is possible because most elderly hold the belief that visting pagodas an socializing would bring them more opportunities and luck in life. Many Vietnamese elderly today go to pagodas to seek peak and tranquillity of mind which may explain why they are more likely to develop a more positive attitude towards life than others. According to Luong, scores of QoL for those who visited pagodas on a regular basis were 4.9 times as high as those who did not [25]. Our findings also accord with the assessment of other studies in England and Bangladesh [10, 27]. Based on the analysis of factors associated with QoL, intervening programs for the elderly should be developed to encourage their active involvement in social activities like visiting pagodas, socializing or joining clubs, and other activities.

## Limitations

As this is a cross-sectional study, it may have precluded the ordering of causality to a certain extent. It is therefore difficult to determine temporal relationships between the predictors and QoL. Another limitation would be related to the QoL measure (WHOQOL-BREF) which has not been widely applied in Vietnam. Yet, we piloted and validated this tool in our research with relatively good internal consistency and validity (both Cronbach's $\alpha$ and b's were relatively high). Besides, on the course of the survey, we had some questions asking for the subjects' past experience which may result in recall bias. However, this study was designed with a survey on a fairly large, representative sample with anonymous and confidential commitment, it would partly reduce that bias. As the majority of studies previously conducted in Vietnam and around the world focused mainly on QoL and its related factors among the elderly in general, our research aims to provide a fundamental understanding about QoL between genders along with its associated factors and make a solid foundation for further research on this topic.

## Conclusions and implications

In this study, there were notable differences in QoL and predictors between elder women and men. Contributors to QoL including 80 years of age and older, and sickness in the past 6 months were significantly correlated with lower QoL in both sexes.

There were other factors related to the QoL of elderly men. Those who followed Buddhism and Christianity were more likely to have a favorable QoL than those who did not. The good social connections they had, the higher their scores of QoL were. As for elderly women, who completed secondary level or above, had medium and high socioeconomic status were more likely to have better QoL than others.

These findings suggest some strategies to improve QoL in the elderly. First, intervention programs need to take gender issues into account due to differences in QoL related factors between men and women. To be specific, local authority, civil and mass organizations should design interventions that help to develop to promote social connections in elderly men in order to improve their QoL. For elderly women, local governments are anticipated to invest more on the development of their educational levels which is greatly expected to give women an edge in promoting their well- being both mentally and physically, which in turn improve their QoL. This is especially integral in rural areas where any advance in academic background will possibly enhance people's awareness on their standing which is of great value to a desirable QoL. In both genders, the elderly who are 80 years of age and over, and experience sickness in the past 6 months need relevant support by healthcare and social organizations to gain more access to medical and social service in case of difficulties and ailments. Besides, State officials and organizations are highly anticipated to provide duly care for the lone elderly, elderly women and those who do not have income. The elderly need to be encouraged to exercise more and participate more in family and social activities. Local clubs for the elderly deserve public attention to be consistently developed with more diversified and enriching activities to encourage socializing, knowledge enhancement, and cultural exchange among members. As Vietnam has much in common with other developing countries in Southeast Asia, we immensely hope that this research could be of great help to public heath systems in similar countries. Moreover, a longitudinal study is needed to address this concern, especially needed are studies of gender, the role of family, marriage and social networks for the elderly QoL.

## Recommendations

The education branch needs to have policies to ensure gender equitable young people in access to education and where possible educational facilities for middle-aged people to catch up.

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The authors declare that they have no competing interests.

## Authors' contributions

TNV designed this study conducted, data collection and statistical analyses, wrote and revised the manuscript. HNV provided technical guidance to the design analysis. TND, TNV and PTN edited the language and technically revised the manuscript. All authors contributed and approved the final version of submitted manuscript.

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ORIGINAL ARTICLE

# Changing the smoking habit: prevalence, knowledge and attitudes among Umbrian hospital healthcare professionals

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Keywords

Tobacco smoking • Prevalence • Health personnel • Health promotion • Hospital

Summary

**Background**. *Health care professionals should work against smoking habit to promote a correct life style. This study aimed to evaluate smoking prevalence and attitudes towards tobacco among Umbrian hospital professionals in a period between 2006 and 2015, since the approbation of the law that ban smoking in hospitals and all public areas in 2003.*
**Methods**. *A cross-sectional study was carried out using a questionnaire administered in 2006, 2011 and 2015 to healthcare professionals. It consists of 53 multiple-choice questions. Potential predictors of current smoking habits were evaluated using univariate and multivariate logistic regression.*
**Results**. *The sample included 475 healthcare professionals. Current smokers constituted 34.53% of the sample and no significant difference (p = 0.257) emerged in prevalence over time (33.74% in 2006; 36.02% in 2011 and 33.77% in 2015). The risk of being a smoker increased by not considering the smoking habit as the main cause of preventable deaths (OR = 2.25; 95% CI: 1.47-3.45). The strongest risk factor, which was significant in both models (p < 0.01), was being against the "No Smoking" law (OR = 18.90; 95% CI: 2.43-147.71; adjusted OR = 22.10; 95% CI: 1.85-264.78).*
**Conclusions**. *The hospital staff has higher prevalence of smoking than the general population. The No Smoking law alone has been shown to be inadequate. Effective results can be achieved only by a common strategy and shared intervention programmes that are based on a workplace health promotion strategy. That for the moment has demonstrated to give interesting outcomes in modifying deep-rooted behaviour patterns.*

## Introduction

According to the World Health Organisation (WHO) the smoking habit causes 10% of adult deaths and is the second cause of preventable deaths. Although the prevalence of smokers has dropped worldwide since 1980, the number of smokers has risen markedly as the population has increased [1]. There were 10.9 million smokers in Italy in 2015 (6.3 million males, 4.6 million females, prevalence 20.8%) [2]. As the WHO indicated, anti-smoking laws are one of the most efficacious public health strategies in the fight against smoking. Laws that protect non-smokers from passive smoking i.e. No Smoking areas, have beneficial effects on smokers and non-smokers. Italy has long been to the forefront in Europe in protecting non-smokers and with art. 51, Law No. 3 dated 16th January 2003, otherwise known as "Safeguarding the health of non-smokers", Italy extended no-smoking areas to all indoor areas except private homes and areas that are specially reserved for smokers. Smoking was forbidden in hospitals by Law No. 584 dated 11th November 1975. The law on "No smoking in certain places and on public transport" had established that smoking was prohibited only in some places like hospital wards, school classrooms, station waiting-rooms and other indoor spaces that were used by the public. Despite specific laws and precise regulations Italian hospitals still cannot be called smoke-free [33]. The present study aimed as assessing how the smoking habits of healthcare professionals in Umbrian hospitals changed 3, 8 and 12 years after implementation of art. 51, Law No. 3 dated 16th January 2003, which prohibited smoking in indoor areas that are open to the public.

## Methods

A multi-centre, cross-section, observational study was conducted in hospitals under the management of Local Health Agency 1 (ex ASL 1) in Umbria. In March 2006, March 2011 and March 2015, healthcare professionals replied anonymously to a questionnaire that had previously been validated in an Italian National multi-centre study, published in 2010 [3].

### The questionnaire

The questionnaire included 53 open and closed questions which were divided into seven sections:

- section 1 asked about demographics with questions on age, gender, job and Health service workplace;
- section 2 assessed what the responder knew about smoking, asking for example about diseases caused principally by smoking and the main reasons why it was worth forbidding smoking in hospitals;
- section 3 assessed the healthcare professional's attitude to anti-smoking legislation and to colleagues who broke the law;
- section 4 assessed the workplace by investigating whether "no smoking" notices were posted and what rooms colleagues used for smoking;
- section 5 monitored smoking-related clinical activity by analysing patient-related actions;
- section 6 inquired exclusively about the healthcare professionals' smoking habits;
- section 7 was answered only by current smokers and asked questions about how often they smoked, attempts to stop smoking, etc.

### Statistical analysis

Descriptive statistics was performed using frequencies, percentages, frequency tables for categorical variables and mean ± standard deviation (SD) for quantitative variables. Non-parametric Mann-Whitney Test was performed to compare continues variable with no normal distribution. Categorical variables were evaluated by chi-square analysis or Fisher's exact test were appropriate. To test the goodness of fit for the logistic regression model the Hosmer and Lemeshow test was performed. All the variable including in the final model had a P-value < 0.25 (not for the administrations).

All the estimates are obtained after Multivariate Imputation by Chained Equations (MICE) approach to handle missing values [4]. This method has three steps. First, for each variable with missing values, a regression equation is created. This model includes the follow-up time and other model covariates. For binary variables this was a logistic regression, and for ordered categorical variables, an ordinal logistic regression. Once all such regression equations are defined, missing values are replaced by randomly chosen observed values of each variable in the first iteration. For subsequent iterations, missing values are replaced by a random draw from the distribution defined by the regression equations. This was repeated for 500 iterations, the final value being the chosen imputed value. This is similar to Gibbs sampling [5]. This entire process was repeated 100 times, thus creating 100 imputed data sets. The next step was to estimate the model for each of these data sets. Finally, the model coefficients are averaged according to Rubin's rule [6]. This ensures that the estimated standard error of each averaged coefficient reflects both between and within imputation variances, giving valid inferences. Univariate estimates under the Logit model are shown in the first column, multivariate estimates are shown in the second colon. A p-value of less then 0.05 was consider to be statistically significant. After estimation we recognize average predicted probabilities (APP) based on logistic regression (not in table).

Statistical analysis were performed with STATA 14.1 (StataCorpLP, Collage Station TX, USA).

## Results

The sample included 475 healthcare professionals, 163 of whom responded to the questionnaire in 2006, 161 in 2011 and 151 in 2015.

Descriptive statistics for the dataset we analyse are reported in Table I. This table summarises the percentage of current smokers in different category.

Females constituted 62.32% of the total. Mean age was 41.78 years (range: 20-66). Doctors made up 16.21% of the sample, 4.63% were medical or nursing students, 43.79% were nurses or ward auxiliaries and 35.37% were other healthcare professionals or technicians.

Current smokers and ex-smokers constituted respectively 34.53 and 24.21% of the sample population. No significant difference (p = 0.257) emerged in prevalence of smokers over time (33.74% in 2006; 36.02% in 2011 and 33.77% in 2015). Current smokers included doctors (12.80%), students (6.10%), nurses or ward auxiliaries (42.68%) and technicians or other professionals (38.41%). Mean age at smoking cessation was 30.8 ± 7.88 years (males 31.25 ± 8.36; females 30.41 ± 7.51).

In the sample population of health workers 50.53% identified the smoking habit as the principal cause of preventable deaths in Italy. A significant (p = 0.004) difference emerged over time as 57.06% in 2006 dropped to 38.41% in 2015. Under half the sample population (43.58%) believed smoking was more dangerous than industrial or traffic pollution. Almost all health workers recognized that smoking was a major risk factor for respiratory and cardiovascular diseases (97.47% and 93.05% respectively); 99.16% knew that smoking was forbidden by law in public places and 97.05% were aware that passive smoke was a health hazard. Even though 57.89% of health workers considered their life-style was a behaviour model for the general population, a significant (p = 0.005) difference emerged over time as the percentage fell from 58.28% in 2006 to 54.97% in 2015. The argument that smoking is harmful and seriously damages health was used both to attempt to dissuade patients and health workers from smoking (94.74%) and to eliminate smoking from hospitals (71.79%).

Even though the majority (92.84%) of the sample population agreed with the law that prohibited smoking in hospitals, significant (p < 0.001) differences emerged over time. In 2006, 95.09% agreed with the new law, rising to 96.27% in 2011 but then dropping sharply to 86.75% in 2015. According to 93.47% of the sample, whoever breaks the law should be penalized but faced with a colleague who smoked only 1.05% take steps to ensure the law was enforced, 29.26% walk away and 29.89% say nothing.

Even though "No Smoking" signs were noticed by 96.21% of healthcare professionals in their Units, 26.95% "often" saw their colleagues smoking in the

**Tab. I.** Frequency distribution of the characteristics for smokers.

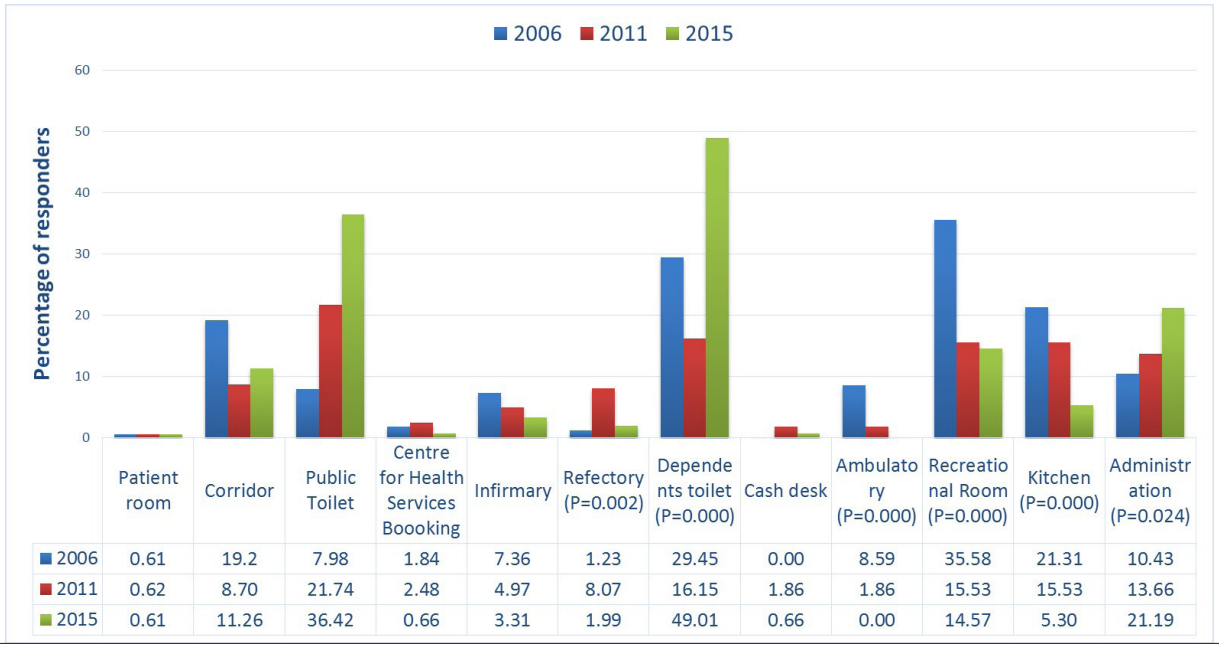
| | Variables (N) | Current smoking total percentage (95%CI) | (p-value) |
|---|---|---|---|
| Gender | Male (179) | 32.96 (26.13 - 40.36) | 0.577 |
| | Female (296) | 35.57 (30.02 - 41.22) | |
| | Missing | - | |
| Profession | Medical doctor (77) | 27.27 (17.74 - 38.62) | 0.298 |
| | Student (22) | 45.45 (24.39 - 67.80) | |
| | Nurse and auxiliary employees (208) | 33.65 (27.27 - 40.51) | |
| | Technician and others (168) | 37.50 (30.16 - 45.29) | |
| | Missing | - | |
| Operative Unit | Medical departments (75) | 34.67 (24.04 - 46.54) | 0.932 |
| | Surgical departments (93) | 35.48 (25.83 - 46.09) | |
| | Other (290) | 33.79 (28.37 - 39.55) | |
| | Missing (17) | 41.18 (18.44 - 67.07) | |
| Behavioural model (for the population) | Yes (275) | 28.36 (23.11 - 34.09) | **< 0.001** |
| | No (150) | 47.33 (39.13 - 55.64) | |
| | Don't know (45) | 33.33 (20.00 - 48.95) | |
| | Missing (5) | - | |
| Tobacco use is the most preventable cause of death | Yes (240) | 25.83 (20.42 - 31.86) | **< 0.001** |
| | No (159) | 44.03 (36.17 - 52.10) | |
| | Don't know (71) | 42.25 (30.61 - 54.56) | |
| | Missing (5) | 40.00 (5.27 - 85.36) | |
| Favour the law banning smoking in hospital | Yes (441) | 31.75 (27.42 - 36.31) | **< 0.001** |
| | No (11) | 90.91 (58.72 - 99.77) | |
| | Don't know (19) | 63.16 (38.36 - 83.71) | |
| | Missing (4) | 50.00 (6.75 - 93.24) | |
| Favour penalties for smokers in hospital | Yes (444) | 33.11 (28.74 - 37.70) | **< 0.027** |
| | No (28) | 53.57 (33.87 - 72.49) | |
| | Missing (3) | 66.67 (9.43 - 99.16) | |
| Attitudes towards colleagues that smoke | Make sanction (3) | 33.33 (0.84 - 90.57) | **< 0.001** |
| | Make him/her move (65) | 30.77 (19.91 - 43.48) | |
| | Give a warning (12) | 25.00 (5.50 - 57.18) | |
| | Move away (107) | 20.56 (13.36 - 29.46) | |
| | Exhort to stop (123) | 11.38 (6.36 - 18.36) | |
| | Say nothing (142) | 61.57 (53.45 - 69.98) | |
| | Missing (23) | 69.57 (47.08 - 86.79) | |
| Administration | 2006 (163) | 33.74 (26.53 - 41.55) | 0.886 |
| | 2011 (161) | 36.02 (28.62 - 43.95) | |
| | 2015 (151) | 33.77 (26.29 - 41.91) | |
| Bold indicates $p < 0.05$. | | | |

hospital ("never" 22.11%; "seldom" 18.11%; "occasionally" 32.42%). Most were found smoking in the staff toilets (31.16%). (Fig. 1) Interestingly, smoking in toilets and offices increased over time while smoking in corridors, kitchens and community rooms dropped.

During case-history recording 24.84% of health care workers declared patients were not asked about their smoking-habit. Only 14.32% stated that detailed information about the patient's smoking habit was elicited e.g. how many years the patient had smoked, how many and what type of cigarettes, when the smoking habit started and stopped, attempts to quit smoking and exposure to passive smoke. Significant ($p = 0.007$) intra-Unit differences emerged in case-history taking. Details on the patient's smoking habit were recorded in 19.35% of Surgery Units and in only 4.00% of Medical Units. An anti-smoking centre existed in the hospital where they worked according to only 10.74% of healthcare workers and a person had been tasked with ensuring conformity with laws on "No Smoking" in public places according to 72.42% of hospital staff.

Healthcare professionals started smoking at a mean age of 18.10 ± 4.45 years. The average age of smokers is 39.94 (range 20-62). They smoked a mean of 11.12 ± 6.72 cigarettes daily. Their smoking habit has changed over the past 4 years as 21.19% of current smokers smoke fewer cigarettes every day, 48.34% smoke the same number and 30.46% smoke more. There was no change over time

**Fig. 1.** Hospital areas where healthcare professionals smoked at the three administrations.




| | Patient room | Corridor | Public Toilet | Centre for Health Services Boooking | Infirmary | Refectory (P=0.002) | Dependents toilet (P=0.000) | Cash desk | Ambulatory (P=0.000) | Recreational Room (P=0.000) | Kitchen (P=0.000) | Administration (P=0.024) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2006 | 0.61 | 19.2 | 7.98 | 1.84 | 7.36 | 1.23 | 29.45 | 0.00 | 8.59 | 35.58 | 21.31 | 10.43 |
| 2011 | 0.62 | 8.70 | 21.74 | 2.48 | 4.97 | 8.07 | 16.15 | 1.86 | 1.86 | 15.53 | 15.53 | 13.66 |
| 2015 | 0.61 | 11.26 | 36.42 | 0.66 | 3.31 | 1.99 | 49.01 | 0.66 | 0.00 | 14.57 | 5.30 | 21.19 |

in the 45.10% of hospital workers who smoke inside the hospital (43.47% males; 56.53% females). Open-air areas like balconies, courtyards and entrances were preferred by 80.92%. In areas where smoking is forbidden 18.42% declared they found it hard not to smoke. 43.06% of healthcare professionals smoke while wearing their uniforms and 40.32% of them feel embarrassed doing it in front of patients or the general public while it made no difference to 59.68%. With regards to wanting to stop smoking, 12.58% said they were willing to try, 19.20% said they had never thought about it, 36.42% claimed they had often thought about it and 31.79% had thought about it sometimes. A majority of health care workers (56.86%) thought there was no effective method to stop smoking. Methods that were considered useful included group therapy (26.79%), pharmacological intervention (22.87%), training courses (19.60%) and penalties (7.18%).

Table II lists the estimated odds ratio when the dependent variable is "smoking at this time".

Gender, profession and operative unit are not significant in univariate model (not showed).

The univariate model showed the risk of being a smoker dropped significantly ($p < 0.01$) as age increased (OR = 0.97; 95% CI: 0.95-0.99) (Tab. II). A significant ($p < 0.01$) major risk factor in both models was not considering the behaviour of healthcare professionals as a model for patients (OR = 2.26; 95% CI: 1.49-3.43; adjusted OR = 1.99; 95% CI: 1.21-3.26). The risk of being a smoker was increased by not considering the smoking habit as the main cause of preventable deaths (OR = 2.25; 95% CI: 1.47-3.45). The strongest risk factor, which was significant in both models ($p < 0.01$), was being against the "No Smoking" law (OR = 18.90; 95% CI: 2.43-147.71; adjusted OR = 22.10; 95% CI: 1.85-264.78). Interestingly, disagreeing with penalties emerged as a risk factor in the univariate analysis (OR = 2.40; 95% CI: 1.11-5.15) but lost significance and positivity when other co-variables were inserted. With regards to the attitudes of healthcare professionals when they saw colleagues smoking, the greatest risk of being a smoker emerged in individuals who said nothing (OR = 11.70; 95% CI: 6.12-22.37; adjusted OR = 10.64; 95% CI: 5.45-20.76) and in individual who walking away (OR = 3.23 95% CI: 1.51-6.91; adjusted OR = 3.27; 95% CI: 1.48-7.23), compared with individuals who suggested stopping.

## Discussion

The main finding of the present study was that the prevalence of smokers is higher among healthcare professionals than among the general population in Italy. In Umbria 34.53% of the population are current smokers and 24.21% are former smokers compared with an Italian national prevalence of 20.80% smokers and 12.10% ex-smokers [2]. Even more remarkable is the lack of significant change over time. Many studies that analysed the smoking habit among doctors and hospital workers confirmed an approach was needed to reach the objective of eliminating smoking with health service buildings and provide greater support for professionals to stop smoking. [7-25]. Analysis of Umbria hospital data showed that in the past ten years the prevalence of smokers has not changed significantly ($p = 0.257$), remaining high in all three observation time-points. Consequently, not only has the "No Smoking" law been broken but it has had no effect on the smoking habit of healthcare professionals. One might argue, as others have, that the "No Smok-

**Tab. II.** Estimated under logistic regression models for the outcome to be smoker.

| Explanatory variable | Cathegory variable | Univariate Logistic Imputed Model (Odds ratio) | Multivariate Logistic Imputed Model (Odds ratio) |
|---|---|---|---|
| Age | | 0.97 (0.95-0.99)** | 0.98 (0.95-0.99)* |
| Behavioural model | Yes (ref) | - | - |
| | No | 2.26 (1.49-3.43)** | 1.99 (1.21-3.26)** |
| | Don't know | 1.27 (0.64-2.49) | 0.68 (0.29-1.60) |
| Tobacco use is the most preventable cause of death | Yes (ref) | - | - |
| | No | 2.25 (1.47-3.45)** | 1.86 (1.013-3.06)* |
| | Don't know | 2.17 (1.25-3.76)** | 1.54 (0.77-3.08) |
| Favour the law banning smoking in hospital | Yes (ref) | - | - |
| | No | 18.90 (2.43-147.17)** | 22.10 (1.85-264.78)** |
| | Don't know | 3.64 (1.40-9.46)** | 4.19 (1.25-13.97)* |
| Favour penalties for smokers in hospital | Yes (ref) | - | - |
| | No | 2.40 (1.11-5.15)* | 0.74 (0.28-1.96) |
| Attitudes towards smoker colleagues | Exhort to stop (ref) | - | - |
| | Make sanction | 3.68 (0.30-43.80) | 1.23 (0.04-33.64) |
| | Move him away | 3.23 (1.51-6.91)** | 3.27 (1.48-7.23)** |
| | Admonish | 2.39 (0.58-9.88) | 2. 19 (0.49-9.81) |
| | Move away | 1.97 (0.94-4.09) | 1.80 (0.85-3.80) |
| | Say nothing | 11.70 (6.12-22.37)** | 10.64 (5.45-20.76)** |
| Administration | 2005 (ref) | - | - |
| | 2011 | 1.10 (0.70-1.74) | 1.02 (0.59-1.76) |
| | 2015 | 1.00 (0.62-1.59) | 0.98 (0.55-1.76) |

Confidence interval in bracket is at 95% significant level. Significant level as follow * $p < 0.05$; ** $p < 0.01$

ing" law's main objective was to protect non-smokers from passive smoke because the damage it causes to the exposed population has long been known [26]. It was estimated in the USA that for every 8 smokers who die of smoking-related diseases, 1 non-smoker died of the effects of passive smoking [27]. Even though the "No Smoking" law in Italy did not impact upon the smoking habit in healthcare professionals, it did at least, in some cases, lead to a change in hospital areas that were used for smoking. In 2013 Principe R. observed that healthcare professionals had at least started smoking in open air areas and were smoking less in indoor areas. At all three observational time-points however, the present study found, as Figure 1 shows, that although fewer hospital workers were smoking in the community rooms, ward kitchens and hospital corridors, more were smoking in staff and public toilets and offices – despite the fact that 99.16% stated they understood the "No Smoking" law, 97.05% were aware of the damage passive smoking caused to health and 97.47% and 93.05%, respectively, knew that smoking was a major risk factor for cardiovascular and respiratory diseases. Since what the healthcare professionals knew about smoking-related health damage and the "No Smoking" law did not impact upon their behaviour patterns the law alone was clearly not enough to ensure smoke-free hospitals.

Interestingly both univariate and multivariate analyses showed the risk of being a smoker increased in individuals who were opposed to the "No Smoking" law. Results were different for responses to penalties. In reply to the question "Do you agree with penalties for people who break the "No Smoking law?" divergent results emerged from the multivariate (adjusted OR = 0.75; 95% CI: 0.27-2.03) and univariate (OR = 2.40; 95% CI: 1.11-5.15) analyses. When estimated with other variables, the high risk of being a smoker in individuals who opposed the "No Smoking" law, and the risk of being a smoker, linked to a weak approval of sanctions, suggested that hospital staff supported the "No Smoking" law in hospitals in theory but preferred not to abide by it in their daily behaviour and not to pay a penalty for breaking the law. These data once again provided evidence that the "No Smoking" law in itself was not enough to make healthcare professionals stop smoking.

The risk of being a smoker was greater among hospital staff who did not think the behaviour of healthcare pro-

fessionals should constitute a model for the general population and staff who said nothing when they saw someone smoking in a No Smoking area. Interestingly, the average predicted probabilities (APP) indicated that 42% of healthcare professionals would be smokers if no one considered doctors as behaviour models and 56% would be if no one reacted upon seeing colleagues breaking the "No smoking" law. The "No Smoking" law has at least created a better working environment for non-smokers and never-smokers and has strengthened the chances of insisting the law be respected, even if they have not completely convinced smokers to uphold it.

In 2010 Callinan reported that the "No Smoking" law created an environment that reduced exposure to passive smoking and supported individuals that wanted to stop smoking [28]. Present data showed that 80.80% of the smokers who replied to the questionnaire had thought about stopping smoking at least once in their lives, 36.43% had thought about it often and 31.79% often. Interestingly, 12.58% were ready to stop smoking. The main problem that is necessary to face is the gap between the numbers of individuals that think about stopping smoking and those that actually do stop. In fact, this study confirms that, a "No Smoking" law alone had no significant effect on the smoking habit of healthcare professionals. It only created limited conditions that supported people who wanted to stop smoking and people who were victims of passive smoking but were unable to oppose to it.

Therefore the "No Smoking" law was neither efficacious nor efficient in promoting health and consequently "different strategies" need to be devised and assessed. In order to have smoke-free hospitals all key elements of the Ottawa Charter should be implemented: Build Healthy Public Policy, Create Supportive Environments, Strengthen Community Actions, Develop Personal Skills and re-orient health services [29]. This implemented in to the workplaces and applied to promote health in specific contexts and to solve "life style issues" like: smoking, low physical activity, stress and high calorie income, has been called Workplace Health Promotion (WHP) [30]. A methodology that the European Network for Workplace Health Promotion contributed to demonstrate to be effective, in particular on these last risk factors [31]. In fact, WHO in 2010, confirmed this approach by the publication "Healthy workplaces, a model for action: for employers, workers, policymakers and practitioners" [32].

## Conclusions

Clearly, much remains to be done in promoting a health culture or, in this case, an anti-smoking culture among workers. At present, the main interventions have been on a legal and political basis. Little has been done on the other key aspects indicated in all models of good practice in WHP. For many years and in many countries no reference to health promotion/protection and workplace safety was found in any training courses for healthcare professionals [30]. Intervening in this area should become a priority in order to create a health promoting culture because healthcare workers, particularly doctors, are the interface with the public in anti-smoking programmes. The Ottawa Charter upholds the workplace as a source of health for the population. No significant results will be achieved unless, starting with healthcare professionals, direct workplace interventions improves behaviour patterns and makes hospitals smoke-free. Targeted strategies such as an anti-smoking centre in every hospital, short consultation service and educational courses for health service personnel may be useful. It is worth noting that only 10.74% of responders knew that an anti-smoking centre existed in the hospital where they were working. Although the main objective of anti-smoking centres is to combat smoking, they are also involved in health campaigns aimed at raising awareness, educating, informing and training healthcare workers and the public and so could usefully become the nerve-centre for integrating diverse strategies. In conclusion, effective results will be achieved only by the implementation of a common strategy and shared intervention programmes that are agreed by all stakeholders: employers, workers, policymakers and practitioners. A stand alone law has been shown to be inadequate to solve deep-rooted behaviour pattern.

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## Authors' contribution

All authors participated in the design, execution and data analysis.

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