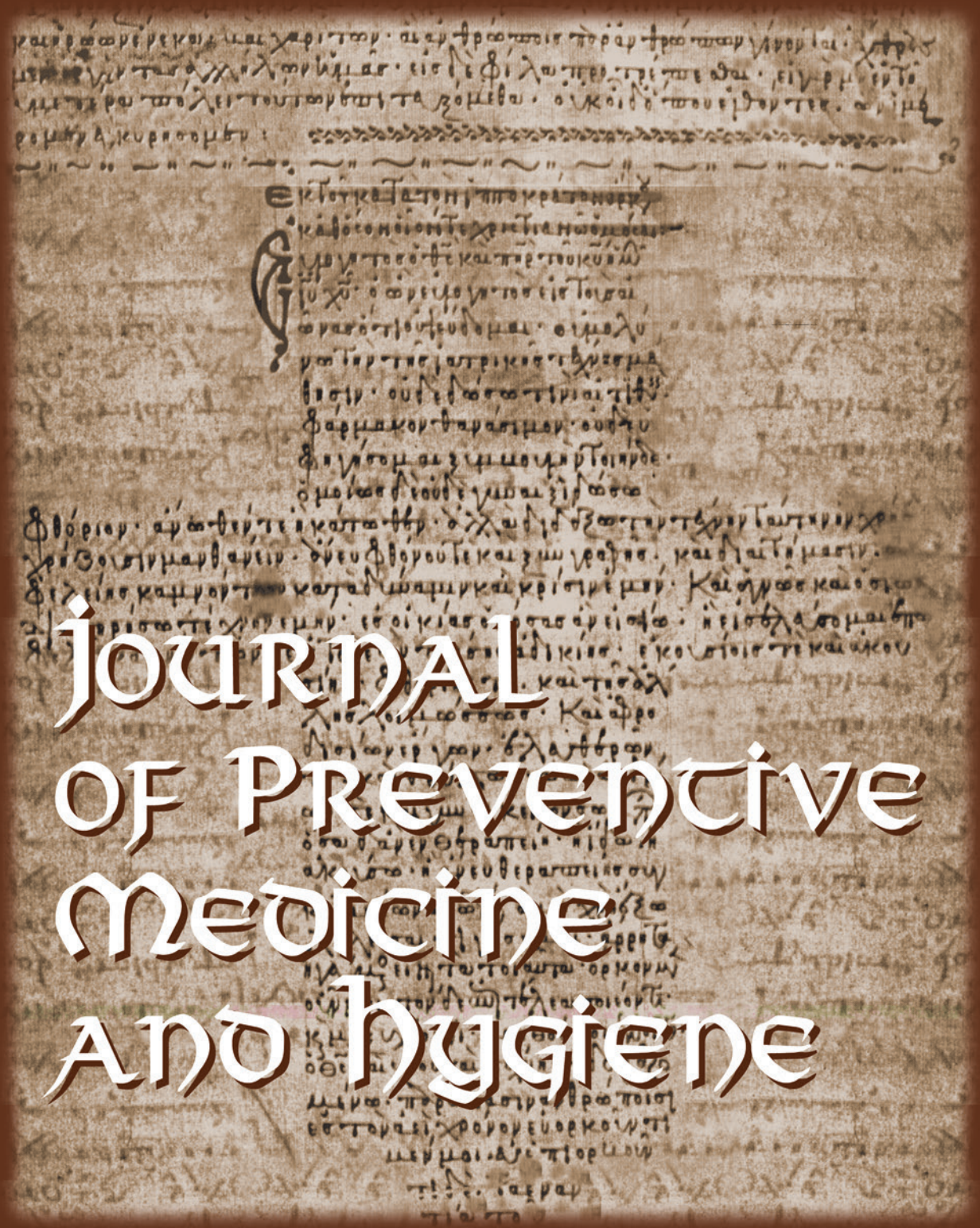
ISSN 2421-4248

vol. 59/3 n.

September 2018

Cited in Index Medicus / Medline
NLM ID 921440 (Pub-Med)



# Journal of Preventive Medicine and Hygiene

The original document of Hippocrates' oath

The Journal has been accreditated, on occasion of the 17th December 2004 Meeting of the Executive and Scientific SItI Councils, by the Italian Society of Hygiene, Preventive Medicine and Public Health

PACINI EDITORE MEDICINA

# JOURNAL OF PREVENTIVE MEDICINE AND HYGIENE

**http://www.jpmh.org**





Managing Editor: Patrizia Alma Pacini
Publisher: Pacini Editore Srl, Via Gherardesca 1, 56121 Pisa, Italy

Published online September 2018

Authorization Tribunal of Genoa, Italy n. 507 - 10/6/1960
Journal registered at "Registro pubblico degli Operatori della Comunicazione" (Pacini Editore srl registration n. 6269 - 29/8/2001).

CONTENTS

Overview

# Prevention of pertussis: from clinical trials to Real World Evidence

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Keywords

Pertussis • Pertussis prevention • Pertussis vaccines

Summary

*Pertussis, a highly contagious infective disease caused by Bordetella pertussis, was in the past very common among newborns and children, causing significant medical, social and economic issues burden, also due to frequent need of hospitalization and high mortality. Following the introduction of vaccines against pertussis, the burden of the disease dramatically decreased, although nowadays, this disease it is still the most widespread among the vaccine preventable ones. First vaccine formulations were composed with whole cell antigen of Bordetella pertussis and were followed by formulations with acellular antigens (PT, FHA, PRN, FIM), that showed to have similar efficacy and less reactogenicity. In particular, all the acellular vaccines, regardless the number of antigenic component included, demonstrated good immunogenicity in clinical trials and high effectiveness in real world evidence studies. Nevertheless, in the recent years it has been notified an increasing number of cases of pertussis.*

*The most recent evidence demonstrated that for an effective control and prevention of pertussis it is necessary to strengthen vaccination coverage among the whole population, providing primary vaccination to newborns and booster in infancy, adolescence and adulthood every 10 years. Finally, vaccination of women at the third trimester of every pregnancy is the most effective intervention to protect the newborn from pertussis in his first months of life, before developing a protective response after the primary vaccination.*

## Introduction

Pertussis (P) is a highly contagious infective disease caused by the Gram-negative bacterium *Bordetella pertussis* (Bp). Until the 1940s, P was extremely common among subjects of pediatric age, especially younger children; from the healthcare, social and economic points of view, it carried a heavy burden in terms of the number of hospitalizations and deaths [1]. Following the introduction of a whole-cell vaccine (wP) in the 1940s, it was thought that the problem of P had largely been solved. Indeed, the frequency of the disease was markedly reduced, at least in areas where the wP was widely used in the pediatric population. In the United States, for example, where over 265,000 cases had been registered in 1934, the incidence of P fell to about 100,000 cases in 1948 and declined further to 1,200-4,000 in the 1980s [2]. Despite this indisputable success, however, the use of wP did not meet with the consensus that would be expected, either among healthcare authorities or among parents. The fact that some of the vaccine formulations available at the time displayed rather low efficacy undoubtedly aroused a certain skepticism. However, what chiefly hindered the systematic introduction of wP into the pediatric vaccination calendar was the fear that its administration might cause potentially severe adverse events. Over time, many of these concerns, such as the fear that the vaccine could cause chronic severe encephalopathy, were shown to be totally unfounded. Nevertheless, the administration of wP did prove to be associated with the onset of significant local reactions and fever in about 50% of vaccinees [3] and of acute systemic manifestations, such as convulsions and persistent crying, in a small, though not negligible, number of subjects [4]. The result of all this was twofold: on the one hand, compliance with vaccination became very low, and vaccination was even not recommended by some healthcare authorities; on the other, efforts were made to develop new vaccines that would be equally efficacious but which would elicit fewer, if any, untoward side-effects.

Subsequently, it was shown that the administration of some components of Bp, such as pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (PRN) and fimbrial proteins 2 and 3 (FIM), could induce a protective immune response without eliciting any noteworthy adverse events. This evidence led to the formulation and subsequent diffusion of acellular anti-pertussis (aP) vaccines containing from 1 to 5 of these components. Numerous studies have shown that aP vaccines have similar short-term efficacy to that of wP, but greater safety and tolerability [5-10]. Consequently, these vaccines have been endorsed by the international scientific community and, despite their high cost, they have been incorporated into the vaccination calendars of a great many countries, with high levels of vaccination coverage being achieved.

Nevertheless, a few years after the introduction of aP vaccines, several epidemiological evaluations clearly indicated that the incidence of P was slowly, though steadily, rising, and had even reached higher values than those recorded in periods of widespread wP use [11, 12]. This increase was seen in all pediatric age-groups, though it was quantitatively more evident among older children and adolescents and qualitatively more marked in infants in whom a greater proportion of severe cases was noted. The so-called re-emergence of P inevitably prompted the scientific community to investigate the reasons for this phenomenon. The re-emergence of an infective disease, when appropriate and apparently efficacious preventive measures have already been implemented, may be due to several factors. Thus, efforts were made to ascertain whether the observed rise was real, rather than the result of a different modality of diagnosis or reporting of cases. At the same time, research was undertaken to establish whether the problem was directly or indirectly related to the aP vaccines themselves, as a result either of lower vaccine efficacy than that which had initially been demonstrated, or of a change in the microbial target of the vaccine. Finally, researchers tried to discover whether the efficacy of the various aP vaccines available differed, and whether the possible re-emergence of P was in some way related to a particular commercial preparation. Although these questions have not been completely clarified, the information currently available enables us to draw some conclusions that can, at least in part, explain the re-emergence of P, and hence to propose some possible solutions to the problem. The present analysis briefly summarizes what is currently known about this issue.

## Have pertussis cases really increased?

For many years, P was regarded as a typical childhood disease characterized by very specific symptoms – often easily recognizable even by non-experts – especially classic fits of coughing. These were the cases that were reported, which sometimes underwent culture tests of microbiological secretions, and on which epidemiological surveys were based. Over time, however, it emerged that in a non-negligible number of subjects infected by Bp, particularly older children, adolescents and adults, the symptoms were very different: merely a persistent or chronic cough, without respiratory impairment or serious systemic disease. Moreover, it was ascertained that, even in infants, P could have manifestations other than coughing fits, such as, for example, apneic crises [13, 14]. The identification of these cases, which play a key role in the spread of the disease, has inevitably raised the number of cases of P reported and notified to the authorities responsible for epidemiological evaluations, which means that the total number of forms of P diagnosed each year has risen to much higher values than those calculated in the past.

In addition, improvements in laboratory techniques now enable Bp infections to be diagnosed much more easily and rapidly than was hitherto possible by means of classical culture methods. Indeed, current techniques of molecular biology allow Bp to be detected in respiratory secretions and anti-PT antibodies to be detected in saliva within a couple of hours [15-17]. These advances have led to a further increase in the number of cases of P notified.

Nevertheless, some studies have shown that the above-mentioned factors alone are not sufficient to explain the re-emergence of P that has been observed in some countries, albeit to different degrees and in different times. This is clearly demonstrated by one such study, which was conducted by the World Health Organization (WHO) in 19 countries in which data were collected for sufficiently long periods of time on the incidence of P, vaccine administration schedules and vaccination coverage, surveillance methods, the case definition of P, and the type of vaccine used. On applying statistical methods that minimized the impact of greater diagnostic accuracy, it emerged that in five of these countries – Australia, Chile, Portugal, the USA and the UK – the increase in P was real, while in the other 14 countries the rise in the number of cases reported could be explained by cyclical variations in the incidence of the disease and by possible sampling errors. Beyond absolute numbers, the data which seemed to suggest a true quantitative and qualitative increase in P were: the significant rise among infants in severe cases requiring hospitalization or transfer to intensive care or causing death, and the disproportionate increase in forms of P diagnosed in adolescents [18].

## The epidemiology of pertussis in Italy

Before the advent of anti-pertussis vaccination, the mean number of notified cases of P in Italy each year was 21,000. By contrast, the latest report issued by the European Centre for Disease Prevention and Control (ECDC), which refers to the period 2011-2015, quotes a mean of 500 cases per year [19]. Thus, in comparison with the pre-vaccination era, the incidence of P in Italy has fallen by 97.6% as a result of both vaccination and the use of aP vaccines (notified cases in 2015 were 503; Tab. I) [19, 20].

However, the incidence of P is probably underestimated, especially among adolescents and young adults, in whom the clinical picture is milder than in infants and whose symptoms may be confused with those of other respiratory conditions. Moreover, laboratory techniques, which are essential to confirming the diagno-

**Tab. I.** Pertussis cases reported in Italy, 2011-2015.

| Year | 2011 | 2012 | 2013 | 2014 | 2015 |
|---|---|---|---|---|---|
| Number of cases | 516 | 489 | 523 | 670 | 503 |

From European Centre for Disease Prevention and Control, 2015 [19], mod.; Epicentro, 2017 [20], mod.

sis, are not frequently implemented. Furthermore, parents are the main source of the contagion of children, in whom the disease tends to be more severe [21, 22]. Notifications of cases of P by age-class in Italy in the period 1996-2009 are shown in Figure 1.

## Short duration of vaccine-induced protection

The re-emergence of P forced the scientific community to seek possible explanations for the phenomenon. Attention was chiefly focused on the duration of vaccine efficacy and on the eventuality that the emergence of genetically mutated Bp strains in the proteins included in the aP vaccines might have blunted, or even nullified, vaccine efficacy.

What aroused the suspicion that aP vaccines might confer only short-term protection was mainly the fact that a large number of cases of P were diagnosed in adolescents, i.e. several years after the primary vaccination. However, it had long been known that neither the disease nor vaccination, regardless of the vaccine used (wP or aP), conferred permanent protection against P. Indeed, the immunity elicited by vaccination lasts from 4 to 12 years, while the protection acquired after natural infection by Bp lasts 7-20 years (Tabs. II, III, IV) [23-36]. For this reason, several countries have for a long time scheduled an anti-P booster vaccination for pre-school children, i.e. aged 5-6 years.

The risk of contracting P is directly proportional to the time that has elapsed since the last vaccination. Indeed, it has been reported that children who receive the preschool booster at 4 years of age have a more than 2-fold higher probability of contracting P during the subsequent years of school than those re-vaccinated at 5 years of age [37]. These findings are completely in line with the results of a recent Italian study aimed at ascertaining the etiology of persistent or chronic coughing in children. The data gathered indicated that about 20% of the children and adolescents who had been affected by coughing for no apparent reason for at least 15 days was suffering from P. In over 80% of cases, the subjects had been regularly vaccinated with an aP vaccines and had received the preschool booster. In some cases, moreover, the booster had been administered no more than 2-3 years before the onset of the disease [38].

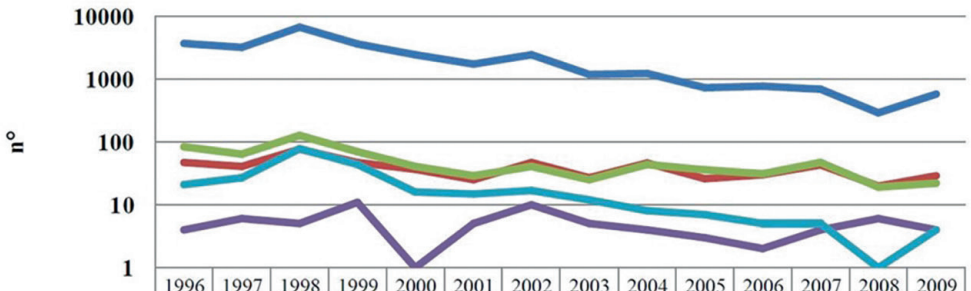
The different immune response elicited by the various aP vaccines, in comparison with that elicited by natural infection or wP, may explain, at least in part, the different duration of the protection induced. Moreover, although the aP vaccines are effective in preventing the clinical manifestations of P, they are unable to prevent colonization by Bp; they therefore do not reduce the risk of transmission from a colonized subject to a healthy subject [39].

**Tab. II.** Studies conducted since the 1990s on the duration of protection induced by the whole-cell pertussis vaccine (wP).

| Author | Year | Subjects | Estimated duration of protection (years) | Country |
|---|---|---|---|---|
| CDC [24] | 1993 | 225 | 4-6 | USA (Massachusetts) |
| Ramsay [25] | 1993 | 3,150 | 8 | UK |
| Nielsen & Larsen [26] | 1994 | Not known | 10 | Denmark |
| He [27] | 1996 | 3,794 | 5-10 | Finland |
| Van Buynder [28] | 1999 | 15,286 | 5-14 | UK |
| Torvaldsen [29] | 2003 | Not known | 6-9 | Australia |

From Wendelboe AM et al., 2015 [23], mod.

**Fig. 1.** Pertussis: trend in notifications in Italy, 1996-2009 (from Gabutti et al., 2012 [22], mod.).




| | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0-14years | 3739 | 3227 | 6689 | 3623 | 2446 | 1730 | 2453 | 1195 | 1236 | 727 | 767 | 696 | 293 | 579 |
| 15-24years | 47 | 41 | 77 | 47 | 37 | 25 | 47 | 27 | 46 | 26 | 30 | 43 | 20 | 29 |
| 25-64years | 84 | 64 | 127 | 70 | 41 | 29 | 41 | 25 | 44 | 36 | 31 | 47 | 19 | 22 |
| >65years | 4 | 6 | 5 | 11 | 1 | 5 | 10 | 5 | 4 | 3 | 2 | 4 | 6 | 4 |
| n.d. | 21 | 27 | 78 | 44 | 16 | 15 | 17 | 12 | 8 | 7 | 5 | 5 | 1 | 4 |

**Tab. III.** Studies conducted since the 1990s on the duration of protection induced by acellular pertussis vaccines (aPs).

| Author | Year | Subjects | Number of Bp components | Estimated duration of protection (years) | Country |
|---|---|---|---|---|---|
| Simondon [30] | 1997 | 4,181 | 4 | 4 | Senegal |
| Tindberg [31] | 1999 | 207 | 2 | 10 | Sweden |
| Salmaso [32] | 2001 | 8,432 | 3 | 3 | Italy |
| Lugauer [33] | 2002 | 10,271 | 4 | 6 | Germany |

From Wendelboe AM et al., 2015 [23], mod.

**Tab. IV.** Studies conducted since the 1990s on the duration of protection following natural infection by Bp.

| Author | Year | Subjects | Estimated duration of protection (years) | Country |
|---|---|---|---|---|
| Wirsing [34] | 1995 | 369 | 20 | Germany |
| Miller [35] | 1997 | Not known (review of studies) | 7-10 | UK |
| Versteegh [36] | 2002 | 4 (case series) | 3-12 | Netherlands |

From Wendelboe AM et al., 2015 [23], mod.

Numerous studies have shown that the levels of antibodies elicited by aP vaccines against the various Bp antigens tend to wane rapidly [40-44]. One of the first studies to investigate this issue was conducted by Esposito et al. [40]. These authors examined 38 children who had regularly received, in the first year of life, the recommended doses of a combined vaccine containing diphtheria, tetanus and hepatitis B vaccines in addition to a 3-component aP vaccine. Analysis of the antibodies against PT, FHA and PRN revealed that, 5 years after the last dose, very few subjects had adequate levels of specific antibodies against all the Bp antigens contained in the vaccine. In addition, *in vitro* study of the response of the peripheral mononucleated cells to exposure to these antigens revealed that a very small number of subjects tested had marked immunological memory.

The different behavior of the aP and wP vaccines can be explained, at least in part, by the fact that each elicits a substantially different immune response. The wP vaccines induce a response that is very similar, albeit less intense, to that induced by natural infection. In both cases, there is a marked production of IgG1, IgG2 and IgG3 antibodies, which is indicative of a significant Th1 response. In addition, there is a considerable Th17 response. The aP vaccines, by contrast, regardless of the number of components they contain, evoke IgG1 and IgG4 production, but elicit a scant Th17 response; this suggests that the aP vaccines induce a mixed Th1/Th2 response. These differences have been confirmed by studies of the $CD4^+$ response. Indeed, in experimental animals, it has been shown that aP vaccines elicit $CD4^+$ which produce large amounts of interleukin (IL)-4 and IL-5, but only a small quantity of interferon (INF)-$\gamma$, a condition that is compatible with a Th2 response [43]. By contrast, the administration of wP is associated with the production of INF-$\gamma$ and IL-17, which suggests a marked Th1 response [32]. In addition, studies conducted on children have shown that the production of $CD4^+$ cytokines indicative of a Th2 response after primary vaccination is markedly higher in aP vaccinees than in those vaccinated with wP [44].

Although the so-called immunological correlates of protection are not as yet available, all these data seem to indicate that the long-term protection induced by aP vaccines may be lower than that provided by wP, which might explain, at least in part, the reemergence of P.

## The appearance of genetically modified *bordetella pertussis* strains

While the presence of genetically different strains of Bp had already been demonstrated in the period when only wP was in use, the phenomenon became more evident after the introduction of the aP vaccines [47-49]. Despite the lack of official data to correlate this microbiological finding with the rise in cases of P, it is nevertheless possible that the two phenomena are related. Indeed, it does not seem irrational to think that the immunological pressure exerted by the vaccine may have favored the selection of mutated strains, especially as the circulation of these strains has been seen to coincide temporally with the widespread use of aP vaccines and the degree of vaccination coverage reached in the various countries [46, 48]. Mutations of the genes that code for the proteins contained in the various aP vaccines have often been detected. In some cases, such as those of some genetic polymorphisms regarding the genes that code for PT, the variation has proved to result in the production of a greater quantity of PT, thereby determining more severe clinical manifestations in the subjects infected. In other cases, such as when one or more genes have been deleted, the practical effect has not been definitively clarified, though it has been supposed that the very lack of a gene that codes for a vaccine protein may limit vaccine efficacy [49-53].

The most significant data on this issue regard PRN. Bp strains lacking this antigen have been detected almost everywhere in the world, though with different frequency. A low incidence has been found in Finland [53], France [54], Italy [55] and Japan [56], while a high frequency has been found in Australia [57], Israel [58] and the United States [59]. In the US, 640 (85%) of the

753 Bp strains identified in 8 states from March 2011 to February 2013, a period of high incidence of P, were PRN-negative. The hypothesis that deletion of the gene coding for PRN may impair the efficacy of vaccines that contain this antigen also seems to be supported by evidence that PRN-negative Bp strains are able to cause very prolonged infections in experimental animals previously immunized with an aP vaccine containing this protein [60]. However, the problem remains open, as the data collected in the field are conflicting. Indeed, retrospective evaluations of the association between the presence of PRN-poor strains and a higher incidence of P have not always shown a positive correlation between the two variables [61].

## The composition of acellular vaccines

All the combined vaccines with Bp antigens contain the PT antigen. The other components of Bp that are sometimes included are FHA, PRN and FIM types 2 and 3. The aP vaccines differ not only in terms of the characteristics of their preparation, such as formulation, combination and concentration in micrograms of the single components, but also with regard to their production modalities, such as the methods of detoxification and purification used. Thus, aP vaccines cannot be compared only on the basis of the number of antigenic components that they contain, not least because the contribution of each antigen to protection is not completely clear [62].
Certainly, the indispensable component, which is always included in all aP vaccines, is PT; this antigen is directly responsible for the development of a protective antibody response following immunization. FHA may be of lesser importance, as it is the strain that is least genetically mutated over time, unlike PRN, the mutations of which have led to the spread of pertactin-resistent strains. By contrast, with regard to the fimbriae, which are also subject to mutations, there is no evidence that they contribute to determining immune protection [19, 22, 62].
Finally, although no correlates or serological indicators of protection regarding P are available, clinical studies have shown that the aP vaccines currently utilized elicit a robust immune response, with post-vaccination antibody levels being higher than on pre-vaccination serological testing [4-10, 23, 25, 30-33, 40, 43, 44, 62].

## Efficacy and effectiveness of the various acellular vaccines

Several studies have assessed the efficacy of the aP vaccines, i.e. the direct and specific efficacy of a single vaccine in preventing P in a given clinical trial [63-75]. Unfortunately, however, the possibility of obtaining results that are truly capable of revealing possible differences among the various aP vaccines is very limited; this is not only because long-term evaluations are lacking, but also because the criteria for defining P have, in many cases, been different, meaning that the types of cases enrolled have been different. Moreover, efficacy studies have directly compared the aP vaccines most commonly administered today, such as the hexavalent, pentavalent and quadrivalent vaccines, for example. In addition, given that the trend in pertussis is determined by a multiplicity of factors, such as the duration of the protection induced by vaccination, the administration of boosters in all age-groups, which is sometimes already implemented, and the risk of natural infection due to the ordinary circulation of Bp, it seems somewhat simplistic to carry out efficacy assessments alone. Rather, in order to draw up the most appropriate strategies for the control and prevention of P, it is important to obtain evidence from studies that assess true efficacy in the field, i.e. effectiveness studies that take into account the contribution of all these factors. Indeed, clinical trials are closed, isolated experiments; as such, they do not take into account such factors as the burden and epidemiology of the disease in a given geographical area, and how these vary over time; nor do they consider the actual implementation of vaccination programs in a given country, i.e. whether coverage targets have been reached, whether booster doses are administered, whether vaccination is scheduled for healthcare workers and pregnant women, and so on. Thus, only studies of effectiveness, i.e. the efficacy of vaccination in real life, can yield real-world evidence, and are therefore essential in order to help policy-makers to plan the most appropriate strategy for the control of pertussis in their own countries.
Table V reports the trials which have evaluated the efficacy of aP vaccines and the surveillance studies in which their effectiveness has been assessed.

### Efficacy studies

Several controlled clinical trials have evaluated the efficacy of aP vaccines in preventing pertussis, according to the definition used in the literature and that proposed by the WHO [74].
In a study by Greco et al. [7], the efficacy of the 3-component aP vaccine was 84% (95% confidence interval [CI] 75.8-89.4). In a trial conducted by Gustafsson et al. [8], in which two aPs were evaluated, the efficacy of the 5-component vaccine was 85% (95% CI 80.6-88.8), while that of the 2-component vaccine was markedly lower: 59% (95% CI 50.9-65.9). This low efficacy score was probably one of the reasons why the 2-component vaccine analyzed in this study was not subsequently registered and was therefore never used in vaccination programs. In a trial conducted by Simondon et al. [63], in which another 2-component vaccine was used, efficacy was 85% (95% CI 66-93); this result was sufficient for approval in the regulatory setting and for implementation in vaccination programs. Finally, in a trial by Trollfors et al., in which a single-component (the PT antigen) vaccine was used, efficacy proved to be 71%; this vaccine is still in use today, especially in northern Europe [65]. In sum, with the exception of the 2-component vaccine that failed to be registered owing to its low efficacy, all the other aP vaccines currently available have displayed high efficacy in the various clinical trials,

Tab. V. Efficacy trials and surveillance studies of the effectiveness of acellular pertussis vaccines (aPs).

| Author or Country | N. antigen components | Efficacy (%) or effectiveness (incidence) |
|---|---|---|
| Greco [7] | 3 | 84% |
| Gustafsson [8] | 2*; 5 | 59%*; 85% |
| Simondon [63] | 2 | 85% |
| Trollfors [75] | 1 | 71% |
| Sweden [65] | 1, 2, 3, 5 | From > 100 cases/100,000 to < 10 cases/100,000 residents |
| Denmark [66] | 1 | From > 100 cases/100,000 to < 10 cases/100,000 residents |
| EU/EEA countries [19] | 1, 2, 3, 5 | From > 100 cases/100,000 to < 10 cases/100,000 residents |
| Non-EU/EEA countries: USA [67], Canada [68], Japan [56] | 1, 2, 3, 5 | From > 100 cases/100,000 to < 10 cases/100,000 residents |

*: 2-component vaccine not registered and never used in national vaccination programs.

and have consequently been used in national vaccination programs. These results show that the efficacy of aP vaccines does not depend on the number of Bp antigen components that they contain; rather, it is determined by other factors, which may be the formulation and production modalities and the methods of detoxification and purification.

Zhang et al. [64] conducted a review of the clinical trials (up to 2014) which had compared the efficacy of wP and aP vaccines, these latter containing from 1 to 5 components. Owing to the methodology adopted, this review also included studies involving vaccines that are no longer produced or utilized in national vaccination programs, having been replaced over the years by vaccines with better efficacy profiles. One of the trials included in the review was that of the above-mentioned 2-component vaccine that was never registered, presumably owing to the low efficacy observed [8]. By contrast, the review did not include the trial of the other 2-component vaccine [63] – which is commonly used today – as it had been conducted according to a methodology that did not fall within the inclusion criteria set by the review.

Moreover, as direct efficacy studies of the aP vaccines commonly used today, such as hexavalent and pentavalent vaccines for pediatric vaccinations, are not available, indirect comparisons have been made on the basis of clinical trials of combined vaccines, for both pediatric and adult use, with different valences, giving rise to a heterogeneity bias. A further bias stems from the methodology used in the clinical trials, particularly with regard to the clinical case definition of pertussis. These limitations and biases were identified and described by the authors of the above-mentioned review. Nevertheless, the conclusions regarding the possible differences in efficacy among aP vaccines containing different numbers of antigens, and the differences between these vaccines and wP vaccines, are inevitably distorted.

### Effectiveness studies

Various national epidemiological surveillance studies have evaluated the effectiveness of anti-P vaccination through the analysis of actual experience in the field. In general, all the currently available 1-, 2-, 3- and 5-component aP vaccines have displayed high effectiveness, yielding a marked reduction in the incidence of pertussis, thanks also to the elevated coverage rates achieved. For example, national epidemiological surveillance in Sweden, which began in the year when aP vaccines were introduced (1996), has shown that these vaccines have great effectiveness; indeed, the incidence of P fell from its pre-1996 level of over 100 cases/100,000 residents to below 10 cases/100,000 residents in the period between 2010 and the last measurement in 2016. Moreover, in an analysis stratified by region and type of vaccine used (1-, 2-, 3- and 5-component), no differences in incidence rates emerged; this indicates that P is effectively under control throughout the nation, regardless of the type of aP vaccine used. Notably, from 1996 to today, the primary vaccination coverage rates in infants (3 doses) have always been 97-98%, i.e. above the 95% target for this age-group [65].

In Denmark, a single-component (PT) aP vaccine has been used since 1995; as revealed by national surveillance, pertussis is well controlled. Indeed, up to the last survey in 2013, the incidence of P always remained below 10 cases/100,000 residents, with the exception of 2002, when an epidemic outbreak raised the incidence to 36 cases/100,000. In this case, too, primary vaccination coverage in infants (3 doses) remained particularly high: 90-99% in the period 1995-2005, 58-91% in the period 2006-2013 [66]. In the period 2013-2016 the pertussis incidence in Denmark was 10.8 cases/100,000 inhabitants [19].

In general, as described in the latest ECDC report, in 2015 the incidence of P in the EU/EEA countries was 9 cases/100,000, a similar value to the preceding years. As expected, it emerged from the various national reports that the incidence was higher among children aged < 1 year: 73.1 cases/100,000; 85% of these cases involved infants below 6 months of age. Precisely on account of this latter finding, the ECDC has recommended that the EU/EEA countries increase their commitment to offering vaccination to pregnant women, since an infant up to the age of 6 months has not yet developed the immunity induced by primary vaccination [19].

Surveillance programs conducted in some non-EU/EEA countries, such as the USA [67], Canada [68] and Japan [56], have also revealed the great effectiveness of aP vaccines in preventing and controlling pertussis, with the incidence of the disease declining to less than 10 cases/100,000, regardless of which aP vaccine is used.

Thus, the WHO's latest position paper on pertussis vaccines states that possible differences among aP vaccines in terms of efficacy, as reported by some trials and re-

views, should be interpreted with caution, in that such findings are contradicted by national surveillance programs and studies of real-world evidence, in which all aP vaccines have displayed great effectiveness in preventing and controlling pertussis [69].

## Strategy for preventing and controlling pertussis (p)

Immunity to P, whether conferred by vaccination or by natural infection, wanes over time; the need to administer booster vaccine doses is therefore essential. Indeed, although the clinical manifestations of the disease become less severe with age, an infected individual may infect infants in their first months of life, when the disease is clinically more serious.

It has been amply demonstrated that the prevention and control of pertussis depend on the achievement of high vaccination coverage rates in the whole population through the adoption of a vaccination calendar with at least the following features [62, 70, 76]:

- scheduled primary vaccination for infants, and administration of a booster dose at pediatric concentration in preschool children;
- a booster dose in adolescents and adults (aged 20 years or more) to be repeated every 10 years, with reduced-concentration vaccines in adults.

The main positions and recommendations of official authorities and international experts are described in Table VI [18, 62, 69, 70, 74, 77].

In addition, recent studies have highlighted the importance of vaccinating specific groups, such as healthcare workers and pregnant women. Regarding this latter group, vaccination with an adult-formulation vaccine in the 3rd trimester of pregnancy confers the protection of the mother's antibodies upon the newborn in the first months of life, i.e. before primary vaccination – a period when infection by Bp may be extremely serious. These studies have revealed an effectiveness of over 90%, which means that more than 9 out of 10 cases of pertussis in infants in the first months of life could be avoided if women were vaccinated in the 3rd trimester of pregnancy [71, 72].

Several countries have already drawn up official recommendations that include the above-mentioned interventions. Nevertheless, it is necessary to strengthen the commitment to implement initiatives aimed at raising public awareness and promoting "vaccine confidence" in the population, in order to achieve full adherence to the recommendations and to reach vaccination coverage targets. Italy, for example, has drawn up the National Vaccination Prevention Plan 2017-2019 (PNPV) [73], which not only envisions both the primary vaccination of infants against pertussis and the administration of periodic booster doses at all ages, but also targets specific groups for vaccination, such as healthcare workers and pregnant women. However, some highly sensitive coverage objectives, such as the vaccination of pregnant women, are still far from being reached, and in some regions are not even actively promoted. By contrast, the vaccination coverage of children of preschool age, has either reached or is close to reaching the 95% objective set by the PNPV for this age-group.

## Conclusions

On the basis of the clinical trials, effectiveness studies and real-world evidence reported in the literature, the following conclusions may be drawn with regard to all

**Tab. VI.** Positions and recommendations of official authorities and international experts on pertussis vaccinations.

| | |
|---|---|
| WHO, Pertussis vaccines position paper [76] | Surveillance studies in countries where aP vaccines (including 1- and 2-component aPs) are used, have shown high levels of effectiveness in preventing pertussis. All aP vaccines have displayed high levels of effectiveness in preventing pertussis, regardless of the number of Bp antigens they contain. |
| WHO, Pertussis vaccines position paper [69] | Differences in efficacy among aP vaccines reported by trials and reviews must be interpreted with caution, as all aP vaccines have shown high effectiveness in national surveillance programs and studies of real-world evidence |
| WHO SAGE Working Group [18] | There is insufficient evidence to conclude that one type of aP vaccine is superior to another. However, the available data underline the importance of reaching and maintaining high coverage rates and of implementing appropriate vaccination schedules. |
| CDC Pink Book [77] | The efficacy of the various aP vaccines varies from 80 to 85%, while their respective confidence intervals overlap, suggesting that no aP vaccine is significantly more efficacious than the others. |
| Martinon-Torres [70] | To achieve high coverage rates in the population, it is necessary to implement a vaccination strategy that includes both the primary vaccination of infants and booster administration in preschool children, adolescents and adults, with particular emphasis on the vaccination of pregnant women. |
| Gabutti [62] | It is important to bear in mind that the use of the current polyvalent aP vaccines has enabled high coverage rates to be achieved and maintained, regardless of vaccine type and the number of Bp components it contains, which is the key factor in successful intervention against pertussis. |
| Zhang [74] | Reviewing clinical efficacy trials alone may yield a limited picture of the benefits of aP vaccines, not least because of differences among the trials themselves and possible biases. |

the currently available aP vaccines, regardless of the number of Bp components they contain:

- they have proved to be highly safe and immunogenic, eliciting a robust antibody response in vaccines, though the correlates of sero-protection against P are not known;
- their efficacy has been demonstrated in clinical trials, i.e. closed experimental studies;
- their effectiveness has been demonstrated by epidemiological surveillance and studies of real-world evidence, i.e. actual experience in the field;
- in countries where wP vaccines have been replaced by aP vaccines, the control of P has proved highly effective;
- strategies for the control and prevention of P must involve the achievement and maintenance of high vaccination coverage rates in the entire population.

Nevertheless, recent evidence has revealed some possible limitations of the currently available aP vaccines, regardless of the number of Bp components they contain:

- the fact that the duration of protection is suboptimal means that Bp carriage cannot be eliminated;
- there is a higher risk of the onset of P in a non-negligible number of subjects, including those who were last vaccinated only a few years earlier;
- booster doses should probably be more numerous and more closely spaced than is currently the case;
- the protection elicited is not associated to a Th1/Th17 response, which is probably necessary in order to achieve more efficacious protection;
- Bp has developed vaccine resistance as a result of several antigenic mutations.

It is to be hoped that future developments will result in the production of pertussis vaccines whose antigenic formulation also takes into account Bp mutations and the possibility of eliciting a Th1/Th17 immune response. In the meantime, however, the results of effectiveness studies and real-world evidence must be taken into account, in order to work out the most suitable vaccination strategies for each specific epidemiological and geographical context.

In general, in countries where aP vaccines are used, as those in Europe but not only, the following interventions and objectives for the prevention of P should be recommended:

- the achievement and maintenance of high vaccination coverage rates at all ages;
- primary vaccination of infants in the first year of life: a schedule beginning at 2 months and using combined vaccines, such as hexavalent vaccines, in order to exploit the synergic effect and achieve important results in the prevention of other diseases, too;
- administration of booster doses in childhood at 5-6 years of age, in adolescence at 12-18 years and in adulthood every 10 years throughout life, and therefore also in the elderly;
- increasing coverage among healthcare workers;
- anti-pertussis vaccination during pregnancy, in order to protect the newborn during the first months of life; this would close the gap which exists in many countries between the official recommendations and actual implementation.

## Acknowledgements

The author thanks Dr. Bernard Patrick for revising the manuscript.
Funding sources: this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Conflict of interest statement

Susanna Esposito received research grants and honoraria for participation in Advisory Boards from GSK, Merck and Sanofi.

## Authors' contributions

SE conceived, designed and wrote this manuscript.

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■ Received on July 16, 2018. Accepted on August 16, 2018.

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ORIGINAL ARTICLE

# Do Tuscan people adhere to meningococcal C vaccination during an emergency campaign?

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Keywords

Meningococcal C invasive disease • Vaccination coverage • Emergency immunization campaign • Population adherence • Tuscany

Summary

**Objectives**. *Tuscany region (Italy) recorded a rise in the number of meningococcal disease cases between January 2015 and February 2016, (52 cases) compared to 2014 (16 cases). The aim of this study was to describe the emergency meningococcal C (MenC) vaccination programme in Tuscany and the population's adherence to the activities performed in the Local Health Unit (LHU) of Florence.*

**Methods**. *The MenC vaccination programme and the planning of the prevention and communication activities were analysed in the LHU of Florence. As an indicator of population's adherence, the vaccination coverage (VC) during the emergency campaign was investigated and adverse drug reactions (ADR) surveillance was reported.*

**Results**. *The communication campaign included a dedicated toll-free telephone number, press releases (newspapers, radio, television, websites), and informative letters addressed to mayors, secondary schools, and sports associations. Citizens aged 11-20 years were the primary target of the campaign. Due to the high incidence of cases among older people, the vaccination was extended to subjects over 45 years. The population's adherence to the vaccination campaign was satisfactory: VC reached 47.1% for the primary target. The ADR reporting rate (3.1/10,000) on meningococcal vaccine in our study confirmed the safety of the vaccination.*

**Conclusions**. *In 2017, only 10 cases of invasive meningococcal diseases (IMD) were reported, suggesting the effectiveness of the immunization campaign. Similar VC during emergency MenC vaccination programmes have been reached in other Italian regions and other EU countries, too. The achievement of greater vaccination coverage is restricted by a sentiment of hesitancy towards vaccines among the general population.*

## Introduction

*Neisseria meningitidis* represents a major cause of meningitis and septicaemia, and it is also a leading cause of morbidity and case fatality rate in all age groups worldwide. Meningococcal disease can lead to permanent sequelae, such as skin scarring, abnormal bone growth, limb loss and multiple amputations, hearing loss, cognitive deficits and visual impairment [1-3]. In Europe, most cases of invasive meningococcal disease (IMD) are caused by serogroups B and C, and the annual notification rate was between 0.55/100,000 population and 1.19/100,000 in the period 2004-2014 [4]. In Italy, the incidence of meningococcal disease was 0.27/100,000 in 2014 [5].

Italian regions can implement different immunization policies and Tuscany was one of the first to recommend meningococcal C vaccination [6]. Since 2005, Tuscany, a central region of Italy with around 3,750,000 inhabitants, has offered the meningococcal C conjugate (MenC) vaccine free of charge at the regional level to all newborns, in the form of a three-dose MenC schedule at three, five, and 13 months of age and a catch-up dose to the children up to six years of age. Since 2008, the MenC vaccine has been offered to all children in a single-dose schedule at 13 months of age, along with the catch-up strategy. This immunization program has proved to be effective in preventing IMD among the birth cohorts involved in the regional offer. Between 2005 and 2014, the number of IMD cases decreased drastically and no cases occurred among the vaccinated subjects [7]. In addition, since 2014, a catch-up dose of quadrivalent conjugate ACWY vaccine has been offered free of charge to adolescents aged between 11 and 18 years who were never vaccinated [8].

However, an increased number of meningococcal disease cases was observed in Tuscany between 2015 and 2016 compared to the previous years. A total of 38 cases occurred in 2015 (31 serogroup C, 5 serogroup B, 1 serogroup W and 1 unknown) and 41 cases in 2016 (30 serogroup C, 8 serogroup B, 1 serogroup W, 1 serogroup X and 1 unknown), while only 16 cases (9 serogroup B, 3 serogroup Y, 2 serogroup C, 1 serogroup A and 1 unknown) had occurred in 2014 [9]. In most cases, molecular typing identified meningococcal serogroup C clonal complex ST-11: a particularly virulent clone of *N. meningitidis* [10].

The unexpected increase in the number of meningococcal disease cases was reported to the surveillance

system. This called for an appropriate public health intervention, including an emergency immunization campaign, which started from April 2015.
The current study was aimed at describing the emergency meningococcal C vaccination programme in Tuscany and the population's adherence to the activities performed in the LHU of Florence – with around 815,000 inhabitants – between January 2015 and February 2016.

## Methods

### Emergency meningococcal C vaccination programme in Tuscany and the planning of prevention activities in the LHU of Florence

Regional deliberations on the meningococcal C vaccination offer were collected for January 2015 - February 2016, and the whole vaccination programme in Tuscany was analysed.
The planning and communication activities of the emergency vaccination programme in the LHU of Florence were deeply analysed and described. In our analyses, we considered the professionals who were involved in the organization and monitoring of the vaccination campaign or in administering the vaccine. Moreover, we also investigated the settings that were selected for the administration of the vaccine.
We also analysed how the need for the vaccine doses to be administered to the target population was estimated and how often the vaccination demand was evaluated to adapt the vaccines supply.

### Data collection, reporting, and the analysis of communication activities and the population's adherence to the emergency campaign

We analysed the communication strategies developed to improve population's adherence. We also evaluated how the vaccine was administered and which professionals were involved in it.
The demand for vaccination, the available slots, and the first date available for vaccination were also investigated.
Immunization coverage was calculated for the age groups involved in the vaccination programme among the resident population between April 2015 and February 2016. Immunization coverage was defined as the percentage of vaccinated individuals among the resident population of the target group and was calculated for the age group initially involved in the vaccination campaign (11-45 years). The vaccination coverage was not calculated for the age group of over 45 years because the period of analysis included less than a month after the recommendation to extend the vaccination offer to older age groups. In fact, during the campaign, the meningococcal C vaccination was even extended to the entire population in particular areas of the region. A filled out consent form was collected from all the vaccines and for those aged more than 55 years additional information were provided: they were informed that no studies were available on the efficacy of the vaccine for people aged over 55 years.
Adverse reactions following vaccine administration were collected and monitored using the adverse drug reaction (ADR) reporting system. ADR records regarding subjects not involved in the target population of the emergency campaign were excluded.

## Results

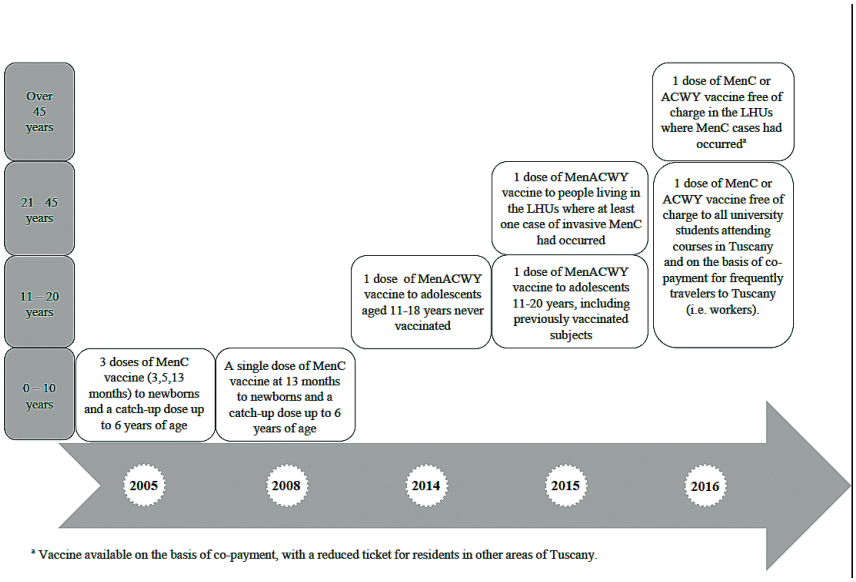
### Regional deliberations and working group

To face the increasing incidence of IMD cases starting from 2015 in Tuscany, one dose of meningococcal ACWY quadrivalent vaccine was offered free of charge to adolescents (11-18 years, including previously vaccinated subjects). Subsequently, the offer was extended to people aged 18-20 and 21-45 living in the LHUs, where at least one case of meningococcal C disease had occurred [11, 12]. Due to the high occurrence of cases among people aged over 45 years, in February 2016, the meningococcal vaccination was extended to all people aged over 11 years free of charge in the LHUs where meningococcal cases have been reported. Particularly, the meningococcal quadrivalent ACWY vaccine was offered to people aged between 11 and 20 years and the monovalent meningococcal C vaccine to people aged over 20 years (Fig. 1) [13].
For citizens aged over 45 years living in the other areas of Tuscany, the meningococcal vaccines were available on the basis of co-payment, with a reduced ticket [13].
To reach the objectives established by the regional health authorities, a multidisciplinary working group was constituted in the LHU of Florence to plan and organize the emergency campaign. In particular, the following professionals were involved: the Health Directorate of the LHU, the Hygiene and Public Health Service Operative Unit (OU), the Responsibles of the Health Service Booking Centre, and the Health Care Assistance OU.

### Planning of the campaign: identification of target population, vaccination settings and healthcare professionals involved

The first step was to quantify the target population of the vaccination programme and the available places for administering the vaccine, both in a hospital setting and in the public health districts.
At the beginning of the campaign, the target population of the vaccination programme consisted of 310,410 people aged between 11 and 45 years in the LHU of Florence. After the extension of the vaccina-

**Fig. 1.** The meningococcal C vaccination programme in Tuscany from 2005 to February 2016.




tion offer to people aged over 45 years, the number increased to 743,128.

The vaccine was administered at 19 vaccination sites: five in hospitals and 14 in health districts over an area of 2,779 km$^2$.

Healthcare professionals working in the public health services administered the vaccines. Each vaccination centre was assigned to a responsible physician, and one or more healthcare assistants were simultaneously involved in the administration of vaccinations.

To guarantee the vaccination offer, additional employees – doctors, nurses, and healthcare assistants – were hired during the monitoring period. Based on shared guidelines, people with a history of allergy or anaphylactic reactions were referred to the Allergy and Clinical Immunology Unit in a hospital care setting, where personnel with expertise in the management of allergic conditions could guarantee additional safety measures.

Moreover, to improve the administration of vaccines, the LHU Health Directorate entered into an agreement with General Practitioners (GPs) and Primary Care Paediatricians. Each physician involved in the campaign had to confirm his/her participation on a voluntary basis via email to a dedicated address. The list of the paediatricians and GPs participating in the vaccination campaign was available on the LHU website. The LHU operators provided the list of unvaccinated patients of the primary target age group (11-20 years) to each GP or paediatrician to increase the immunization coverage of their patients.

As far as primary care was concerned, 88 among 109 primary care paediatricians (80.7%) and 412 among 606 GPs (68%) participated in the emergency vaccination programme.

Moreover, an agreement was established with some voluntary associations to organize vaccination clinics for the volunteers themselves and it was subsequently extended to all citizens.

In addition, the LHU involved their occupational physicians to provide meningococcal C vaccine doses to private companies interested in getting their workers vaccinated.

## Communication activities

The communication campaign with the population included different strategies to improve population's adherence.

Firstly, a dedicated toll-free telephone number, managed by healthcare assistants, was activated to offer information regarding the vaccination programme. If the line was busy, people could leave a voice message with their name and telephone number, to be called later by a healthcare assistant.

During the whole immunization campaign, several press releases – in newspapers and on the radio, television, and websites – were issued at the regional and local levels [14, 15].

Letters containing information regarding the disease and the vaccination campaign were sent to mayors, to secondary schools, local education agencies, and sports associations.

Moreover, vaccine promotion days for voluntary associations were also organized. In particular, the vaccine days were promoted with the support of the Italian Red Cross.

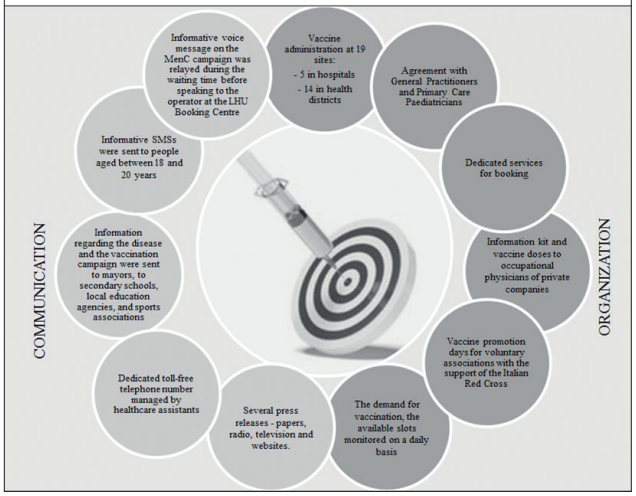
Since October 2015, about 8,000 informative SMSs were sent to people aged between 18 and 20 years who had previously accessed the Booking Centre number. The SMS text was as follows: "LHU of Florence reminds free meningococcal C vaccination for citizens aged 11-45 years". In the same period, an informative voice message on the meningococcal C campaign was

relayed during the waiting time before speaking to the operator at the LHU Booking Centre.
Reminders for vaccination appointments were sent via SMS the week before the scheduled date.
Public health and primary care services also played an important role in the communication strategy. They informed people about the disease, its prevention through vaccination, and the way to access immunization services (Fig. 2).

### Monitoring of vaccination demand, coverage and ADR

The working group for the evaluation of the vaccine campaign monitored the vaccination demand continuously, on a daily basis. The weekly average trend of vaccination demand from April 22, 2015 to February 29, 2016 and the day of the onset of the IMD symptoms have been shown in Figure 3.
Following the first IMD cases, the weekly demand for vaccination increased twofold from the first to the second week of monitoring. The values increased from 538 to over 1,000 weekly contacts.
During summer, the average weekly demand for vaccination reached its lowest level.
The highest values were recorded in February 2016. In week 45, 3,280 daily contacts were registered on an average, with more than 4,000 requests being received on workdays and a peak of 5,334 contacts.
During the monitoring period, the demand for vaccination was zero or very low on weekends or national holidays. Only in the last month of the observation period, even during the weekends, an average of more than 400 contacts was registered.
Public health service professionals recorded vaccinations in a computerized immunization register or in paper forms, while the GPs and Primary Care Paediatricians emailed periodic reports to the LHU staff.
During the evaluation period (April 2015 - February 2016) 64,998 subjects aged between 11 and 45 years (32,053 in the 11-20 age group and 32,945 in the 21-45 age group) were vaccinated, reaching the coverage of 21%. The vaccination coverage obtained in the LHU of Florence and in Tuscany has been reported in Table I.
In the LHU of Florence, 20 adverse reactions in the target population were notified to the reporting system in that period. Among the about 65,000 administered doses, 18 mild reactions (fever, swelling, redness and pain at the injection site, general feeling of discomfort, and headache) were reported. Only two severe reactions, both with complete recovery, were reported to the monitoring system. One case was of convulsion and bradycardia and another of hyperpyrexia and delirium.
The ADR reporting rate in the overall period was 3.1/10,000. The mean age of the people with an adverse reaction was 28 years (range: 11-43 years).

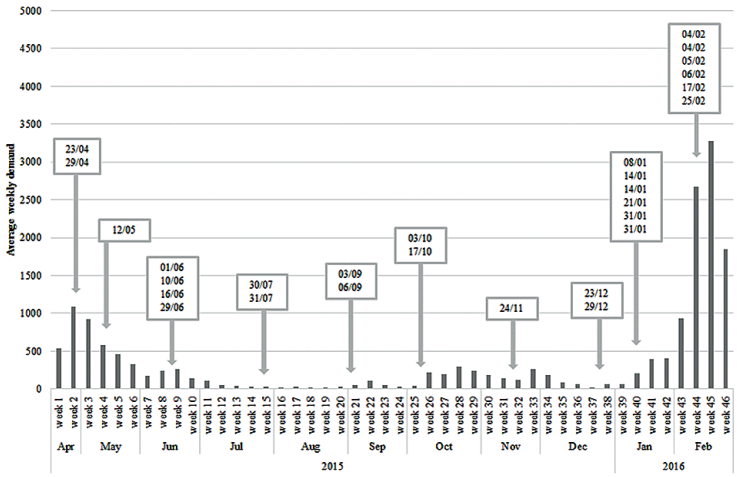
**Fig. 2.** The LHU of Florence vaccination programme. Organization and communication strategies during the emergency meningococcal C campaign in Tuscany.




## Discussion

The regional emergency meningococcal C vaccination campaign was planned and implemented in an extremely short time. Moreover, it was continuously adjusted to the evolution of the epidemiological situation.
The main goal of the vaccination programme was to protect adolescents and young adults – the most susceptible subgroups of the population – and to reduce the bacterial circulation among the general population, by means of the herd immunity effect, through the vaccination of people aged 11-20 years with a single dose of meningococcal ACWY vaccine.
The achievement of high coverage rates among adolescents is an important public health issue, in order to reduce the nasopharyngeal carriage [16]. Conversely, the vaccination offer to older people mostly represents an individual preventive measure.
In 2015, the incidence of meningococcal disease of serogroup C in Tuscany was 0.83/100,000, about three times higher than the previous years. The median age of the cases was 28 years (range 3-82). Most of the cases occurred in people aged 15-20 and 21-30 years, with incidence rates of 3.23/100,000 and 2.83/100,000 respectively [17].
Usually, in the previous years, the highest incidence of IMD was observed in the younger age groups [7, 16]. In Tuscany, a consistent number of cases (28%) has occurred in people older than 50 years since 2015. Of that, 19% has occurred in people aged over 65 years. The increase in the number of IMD cases among adults in Tuscany can be partly explained by the extension of the bio-molecular diagnostic method to adults since 2015 [18].
As expected, the occurrence of each new meningococcal invasive case generated a rise in vaccination demand. In particular, in February 2016, weekly contacts on workdays were almost 3,000 on an average, reaching peaks of over 4,000 requests per day.

**Fig. 3.** The average weekly demand for meningococcal vaccination and the date of invasive meningococcal cases[a] ([a]: the date of the onset of symptoms in the cases have been reported in the boxes) in Tuscany (April 22, 2015 - February 29, 2016).




The doses supply and vaccination demand were checked daily so as to modulate the offer. The vaccine doses availability was monitored to ensure an adequate response to the vaccination demand for the following days. To meet the increased demand for vaccines, the doses were also obtained from abroad and, starting from February 2016, it was decided that the monovalent C vaccine would be used as an alternative to the tetravalent vaccine for people aged over 20 years.

The VC calculated is the most effective measure of the population's adherence to the emergency vaccination campaign. Concerning the period investigated in this study, after 11 months of emergency campaign in the LHU of Florence, as well as in Tuscany, around 21% vaccination coverage was achieved among 11- to 45-year-old people, while almost half of the primary target (subjects aged 11-20) in Tuscany were vaccinated in the observation period. This VC was obtained thanks to interventions targeting adolescents through school-based communication activities, involvement of sports associations and SMS with informative text on the vaccination campaign sent to subjects aged 18-20 years.

Similar emergency meningococcal C vaccination programs have also been planned in the last decades in other Italian regions, such as Veneto, as well as in other EU countries, due to the changes in IMD incidence rates [19-21].

In 2007, a cluster of IMD cases, due to the same clonal complex ST-11, was reported among subjects aged between 15 and 33 years in Veneto. A campaign was started to offer meningococcal C vaccination to all inhabitants aged between 15 and 29 years living in the province of Treviso where the cases occurred. The overall vaccination coverage was 60%, but it decreased to 40% in the older age group [20, 21].

**Tab. I.** The meningococcal C vaccination coverage for people aged 11-20 and 21-45 years in the Local Health Unit of Florence and in Tuscany (April 2015 - February 2016).

| Age group | Vaccination coverage in LHU of Florence | Vaccination coverage in Tuscany |
|---|---|---|
| 11-20 y[a] | 47.1% | 46.3% |
| 21-45 y[b] | 13.6% | 14.1% |
| Total | 20.9% | 21.2% |

[a]: LHU of Florence inhabitants - 68,082; Tuscany inhabitants - 317,911; [b]: LHU of Florence inhabitants - 242,328; Tuscany inhabitants - 1,119,990.

In England and Wales, the meningococcal vaccination campaign consisted of a vaccination offer to all newborns and citizens aged between five months and 17 years. GPs administered the vaccine to children up to five years, while the subjects belonging to the 6-17-year-old age group were vaccinated in schools. The overall coverage achieved through the school-based campaign was at least 85%. The highest vaccination coverage was reached among children aged between 8 and 12 years, while it was the lowest (43%) among 15- to 17-year-olds. Adolescents represent a difficult group to reach for vaccination and the results obtained in Tuscany are similar to those in the UK [22].

Nevertheless, IMD cases continued to occur in Tuscany and some cases were also registered among the vaccinated subjects. The occurrence of IMD in the vaccinated subjects has been reported in other EU countries, too [23]. However, in Tuscany, the fatal cases in 2015 occurred only in the subjects who were never vaccinated or who were vaccinated many years before. Following this evidence and in accordance with confirmation from scientific literature, Tuscany region recommended meningococcal vaccination for all adolescents, included the subjects already vaccinated in childhood [24, 25]. Currently, in Tuscany, meningococcal C vaccination consists of a three-dose

schedule: the first dose at 13-15 months (monovalent vaccine), the second dose at six to nine years (monovalent vaccine), and the third dose at 13-20 years (quadrivalent ACWY vaccine). This strategy is supported by the evidence that meningococcal serogroup C antibody levels wane rapidly after meningococcal conjugate vaccination among young children, leaving adolescents with low antibody levels. Booster doses are needed to sustain antibody levels in teens. Moreover, in Tuscany, the second (or the third) dose with meningococcal ACWY vaccine is guaranteed to subjects aged between nine and 20 years already vaccinated more than five years previously. During the transition to the new vaccination schedule with three doses, vaccination is also assured for unvaccinated subjects aged up to 20 years [26].

Our results described about a year of emergency campaign in the LHU of Florence and the population's adherence to the vaccination offer. When this report was being written, the emergency meningococcal C vaccination campaign in Tuscany was still going on and the number of primary care physicians involved in the vaccination campaign had increased [27].

In 2017, only 10 IMD cases were reported, suggesting the effectiveness of the meningococcal immunization campaign among adolescents and the general population [28]. The achievement of greater vaccination coverage is restricted by a sentiment of hesitancy towards vaccines among the general population, especially as far as the safety issues of vaccinations are concerned. However, the ADR reporting rate on meningococcal vaccine for our study confirms the safety of the meningococcal vaccination, even for adolescents and adults.

## Conclusions

IMD cases in Tuscany sparked alarm among the general population and prompted the health authorities to plan an emergency vaccination and communication program. The preventive activities provided by the LHU of Florence, in agreement with the regional health authorities, represented an example of good practice in the surveillance and control of this particularly frightening disease.

Moreover, the involvement of public health professionals and primary care physicians made possible the widest distribution of vaccinations in the Florentine area, even to the subjects who were the most difficult to reach, such as adolescents and young adults, with satisfactory adherence to the vaccination campaign.

## Acknowledgements

Funding sources: this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Conflict of interest statement

None declared.

## Authors' contributions

AB, DP, FP, make substantial contributions to conception and design, and/or acquisition of data (DP, FP, LB, GM, MGS, TB, SG, EG), and/or analysis and interpretation of data (AB, DP, LB). SB, PB, GB participate in drafting the article or revising it critically for important intellectual content. All authors give final approval of the version to be submitted and any revised version.

Ethical approval: ethical approval was not required for this study, because the vaccination coverage, the demand for vaccination and the ADR data were collected in an aggregated way and properly anonymized. Moreover, information on the onset of Meningococcal C cases were in the public domain (newspaper accounts).

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■ Received on May 18, 2018. Accepted on July 17, 2018.

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ORIGINAL ARTICLE

# HPV vaccine and autoimmune diseases: systematic review and meta-analysis of the literature

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Keywords

Sistematic review • Meta-analysis • HPV vaccine • Autoimmune diseases

Summary

**Background**. *In the literature conflicting opinions are detectable on the onset of adverse events as autoimmune disease post HPV vaccine and often case reports describes the onset of one of these events, but don't emerge a clear relationship and we don't have data to support it.*
**Methods**. *We carried out a systematic review to identify all scientific publications dealing with the correlation between vaccine anti-papillomavirus and new onset of autoimmune diseases. We searched the main scientific databases (PubMed, Sciverse Scopus, Web of knowledge and Cochrane Central Register of Controlled Clinical Trials) for the following search terms: "vaccine"; "anti-papillomavirus"; "autoimmune"; "disease"; "disorder". To evaluate the safety of HPV vaccines, the dichotomous data on the number of subjects experiencing an autoimmune disorder in the study vaccine group and the placebo group were extracted from each study with subsequent determination of the risk ratios and their 95% confidence intervals. We combined data statistically using a random effects model.*
**Results**. *We conduct a meta-analysis on six studies on bivalent and quadrivalent HPV vaccine. The total number of subjects included in the meta-analysis comprised 243,289 in the vaccine group and 248,820 in control groups. Four of the six trials had a Jadad score of 3 or 4 indicating an adequate trial quality. The most frequent autoimmune disease observed across the six studies were musculoskeletal,CNS conditions and endocrinological conditions . The results of the meta-analysis demonstrated no correlation between autoimmune disorders and HPV vaccines (pooled OR 1.038, 95% CI 0.689-1.562).*
**Conclusions**. *No correlation was identified for bivalent and quadrivalent HPV vaccines. It's therefore essential to correctly inform the general population in order to try to increase both Italian and international vaccination coverage.*

## Introduction

Human papillomavirus (HPV) is one of the commonest sexually transmitted viruses worldwide, with initial infections typically occurring soon after sexual debut. Today more than 290 million women have a human papillomavirus (HPV) infection. An effective vaccine is available as part of routine immunization programmes in 65 countries. In low- and middle-income countries, where most cases of cervical cancer occur, if 70% vaccination coverage were achieved the deaths of more than 4 million women would be avoidedover the next decade [1].
There are three vaccines currently available: one bivalent that protects against HPV types 6 and 11 which are the most common causes of genital warts,one quadrivalent, which also provides protection against HPV types 16 and 18 and the last one nonavalent vaccine that contains serotypes 6, 11, 16, 18, 31, 33, 45, 52 and 58.. Despite this availability, the latest HPV vaccination coverage in USA estimates show that only 60 percent of adolescents aged 13-17 years have received one or more doses of HPV vaccine, with a gender gap in HPV vaccination rates (about 65 percent of females having received the first dose of HPV vaccine compared to 56 percent for males) and only 43 percent of adolescents are up to date with all recommended doses of HPV vaccine [2].
The most common reasons given for refusing the HPV vaccine were lack of vaccine endorsement by physicians, lack of perceived need for the vaccine, lack of knowledge and safety concerns and caregivers' concerns about safety or potential side effects [3-6].
One of the frequently attributed effects to the HPV vaccine that is cited in the literature is the onset of autoimmune disease. Alleged associations between HPV vaccinations and autoimmune disorders (ADs) have been reported in the international literature and the most frequently proposed mechanism to account for these is molecular mimicry [7-11].
Objectives: in this study, we want to investigated whether the HPV vaccine is associated with the onset of ADs.

## Materials and methods

### Search strategy and selection criteria

We carried out a systematic review to identify all case control study dealing with the correlation between vaccine anti-papillomavirus and new onset of autoimmune diseases.
This is "a condition in which the body recognizes its own tissues as foreign and directs an immune response

against them" such as gastroenteritis, connective tissue disorders, alopecia, CNS conditions and endocrine autoimmune disease, etc… We searched the main scientific databases (PubMed, Sciverse Scopus, Web of knowledge and Cochrane Central Register of Controlled Clinical Trials for the following search terms: "vaccine"; "anti-papillomavirus"; "autoimmune"; "disease"; "disorder", using the function "AND" and "OR". The bibliographies of all relevant articles, including reviews, were screened for further references. No language restrictions were imposed; papers in languages we were unable to read were translated using Google Translate. We developed the search terms in accordance with the Medical Subject Headings thesaurus, using a combination of test searches and via collaboration between independent researchers and knowledge users. After deleting duplicates, we further screened titles, abstracts, or entire articles using exclusion criteria. Screening was carried out independently by two authors (RS, CG). Any disagreement about eligibility between reviewers was resolved by a third author (VLF). The first two authors extracted data from included papers using a data extraction form reviewed by the other co-authors. These procedures comply with the PRISMA guidelines for reporting systematic reviews [12].

### Data extraction

Two independent reviewers (CG and RS) identified potentially relevant articles and collected the following data: first author's last name; year of publication; clinicaltrials.govidentifier (if applicable); study design; total number of participants; age range; gender; disease background and study arms with number of vaccinated participants in each arm. We found after this process only clinical trial for the purpose of our study,

### Evaluation of study quality

AD outcomes were identified from the included studies and considered for the meta-analysis. We used the Cochrane guidelines for systematic reviews of interventions [13] and two reviewers (CG, RS) independently assessed the quality of individual studies included in the meta-analysis. The Jadad scale for reporting randomised controlled trials (RCTs) was employed. This assigns an overall score of the methodological quality of a study from zero to five [14]. Although studies were not on the basis of this assessment, the quality scores were taken into account when describing results.

### Data analysis

To evaluate the safety of HPV vaccines, the dichotomous data on the number of subjects experiencing an AD in the study vaccine group and the placebo group were extracted from each study with subsequent determination of the risk ratios (RR) and their 95% confidence intervals (CI). We combined data statistically using a random effects model. I2 statistics and test Q di Cochran were used to assess the heterogeneity between the studies included. Values of I2 can be interpreted as low (25-50%), moderate (50-75%), and high (75% and higher) levels of heterogeneity. Meta-analyses were performed using package "meta" rel.4.9 of the software R.

### Dealing with missing data

Our analysis relies solely on existing data.

### Assessment of reporting biases

Due to the limited number of studies available for meta-analysis, assessment of publication bias was not applicable. The review is subject to publication bias.

## Results

A total of 235 references were identified from electronic databases in the search performed on May 3 and 4, 2018 (Fig. 1). Once duplicate entries (116) had been removed, references were further evaluated for inclusion based on the title and/or abstract. 119 potentially relevant articles were thereby included in the next stage for full-text evaluation. These publications included: 3 reviews, 8 animal studies,14 case reports, and 2 were studies regarding treatments; the other studies were position papers, letters comments and replies. The characteristics of the study population, interventions, control groups, the evaluated outcomes and/or design of the study (PICOS) failed to meet the inclusion criteria in 113 publications. Most of these studies were excluded as they did not include a control group. Ultimately, a total of six RCTs fulfilled all inclusion criteria and were selected for the meta-analysis [17-20].

### Study characteristics

The main characteristics of the selected RCTs are summarized in Table I.

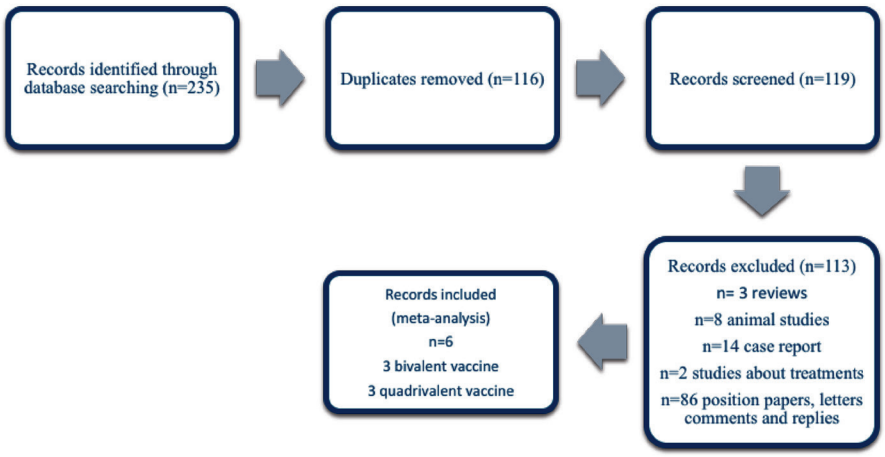
Of the six studies selected, three used a bivalent vaccine and three a quadrivalent vaccine. The six vaccine trials on enrolled a total of 492,109 individuals, 243,289 in the treatment group (i.e. subjects administered vaccines) and 248,820 in the control groups (i.e. subjects receiving another vaccine (such as HBV vaccine, HA vaccine)or no vaccine). The age of the enrolled subjects in the nine studies varied from 9 to 26 years and all studies reported the number of subjects who experienced a specific AD.

In the study by Geier et al. the vaccine adverse event reporting system (VAERS) database was examined for adverse event reports associated with vaccines administered from January 2006 through December 2012 to recipients between 18 and 39 years old with a listed residence in the USA and a specified female gender [15].

In the study by Block et al. females aged 9 to 26 years and males aged 9 to 16 years received at least 1 dose of HPV-6/11/16/18 vaccine or placebo and were studied for all serious and nonserious adverse events (AEFI) s and any new medical conditions were also recorded for the entire study period [18].

In the study by Verstraeten et al. AEFI data were collected prospectively. In addition to a study-specific list of local or general events solicited during a brief period following vaccination, unsolicited AEFI were cat-

Fig. 1. Flowchart of the evaluation and inclusion process for the meta-analysis [21].




Tab. I. Characteristics of the included studies.

| Authors, year | Enrollment | Age range (yrs) | Study arms 1 | Study arms 2 |
|---|---|---|---|---|
| Geier, 2015 | 22,011 | 9-26 | 5124 | 16887 |
| Verstraetena, 2008 | 68,512 | > 10 | 36,744 | 31,768 |
| Block, 2010 | 21,464 | 9-26 | 11,778 | 9,686 |
| Grimaldi-Bensouda, 2014 | 1,365 | 14-26 | 269 | 1,096 |
| Angelo, 2014 | 47,857 | 9-25 | 27,353 | 20,504 |
| Willame, 2016 | 129,937 | > 9 | 64,964 | 64,973 |

egorized as follows: non-serious AEFI, serious AEFI, medically significant events and new onset of chronic disease. These were reported to investigators during a study visit and were collected for 30 days after each vaccine dose [16].

In the study by Grimaldi-Bensouda a total of 113 specialized centers recruited (from December 2007 to April 2011) females aged 14-26 years with incident cases of six types of ADs: idiopathic thrombocytopenic purpura (ITP), central demyelination/multiple sclerosis (MS), Guillain-Barrè syndrome, connective tissue disorders (systemic lupus erythematosus, rheumatoid arthritis/juvenile arthritis), type 1 diabetes mellitus and autoimmune thyroiditis. Control subjects matched to cases were recruited from general practice. Cases and controls were compared with regard to exposure to the quadrivalent HPV vaccine [17].

In the study by Willame et al. 9-25 year-old women after the first AS04-HPV-16/18 vaccine dose were compared to three unexposed cohorts and were observed within the one year follow-up period [20]. In the study by Angelo et al. three groups were considered: adolescent girls/women receiving HPV-16/18-vaccine alone (HPV group), subjects receiving HPV-16/18-vaccine coadministered with another vaccine and subjects receiving no vaccine (HVP group control) and unsolicited AEFI were reported for 30 days after each dose [19].

### Study quality

The methodological quality of the included RCTs was satisfactory (Tab. I), except for those conducted by Geier and Verstraeten et al. Four out of the six studies (66.6%) had a score of 3 or 4 on the Jadad scale. The studies by Geier and Verstraeten et al scored 0 as no information was available about randomized method and blinding of these studies.

These publications included: 3 reviews, 8 animal studies,14 case reports, and 2 were studies regarding treatments; the other studies were position papers, letters comments and replies.

### HPV vaccine vs other vaccine or placebo or no vaccine

The most frequent ADs observed across the six studies were musculoskeletal (e.g. systemic lupus erythematosus), CNS conditions and endocrinological conditions (especially thyroid disease). The results of the meta-analysis demonstrated that ADs were not significantly more frequent in subjects receiving HPV vaccines than in those receiving placebos (pooled OR 1.038, 95% CI 0.689-1.562 (Fig. 2).

The Cochran Q test (67.68; $p < 0.001$; GdL = 5) showed heterogeneity for many characteristics:variability, sample size in individual jobs etc. The Higgins index (92.61%) also showed significant heterogeneity. For this reason, the choice fell into a "random" model with an odds ratio of 1.038 and 95% CI of 0.689-1.562.

Analysis of the forest plot reveals that almost all publications fall on the line of no effect, except for the study by David A. Geier (OR = 2.186; CI 95% 1.757-2.720), or 2.65% of vaccinated subjects with autoimmune dis-

ease versus 1.23% of unvaccinated subjects with autoimmune disease.
The funnel plot also showed a wide variability in the data (Fig. 3).
In the study by Geier et al. was observed that cases of autoimmune disease (such as gastroenteritis, arthritis, systemic lupus erythematosus, vasculitis, alopecia or CNS conditions) were significantly more likely than controls to have received the HPV4 vaccine. Casess with Guillain-Barre syndrome or thrombocytopenia were no more likely than controls to have received the HPV4 vaccine [15]. In the study by Verstraeten et al. the autoimmune events observed included thyroid disease (the most common ), LES, and neuroinflammation (multiple sclerosis and optic neuritis). For each disease category or for any individual event, most relative risks were close to 1 and all the 95% CIs included. The overall relative risk was 0.92 (95% CI: 0.70, 1.22). The highest relative risk for an individual event was 2.39 for systemic lupus erythematosus and the lowest were 0.53 for diabetes mellitus and nephritis. The 95% CIs of the relative risks of these events all included 1, suggesting no significantly increased or decreased risk following administration of the HPV-16/18 vaccine [16].
In the study by Block et al. no significant difference in AD rates were noted between vaccine and placebo recipients The most common autoimmune conditions were arthralgia, hypothyroidism and psoriasis [18].
In the study by Grimaldi Bensouda et al. there was no evidence of an increased risk of the studied ADs following vaccination with Gardasil within the time periods studied. The ADs included idiopathic thrombocytopenic purpura, central demyelination/multiple sclerosis, Guillain-Barrè syndrome, connective tissue disorders, type 1 diabetes mellitus and autoimmune thyroiditis [17].
In the study by Willame et al. the odds ratio (OR) (95% CI) of ADs was 1.41 in female and 1.77 in male cohorts when compared to the respective female and male historical cohorts. Secondary endpoints were evaluated for the following diseases with > 10 cases: Crohn's disease (OR: 1.21 for female and 4.22 for male cohorts); autoimmune thyroiditis (OR: 3.75 for female and no confirmed cases for male cohorts) and type 1 diabetes (OR: 0.30 for female and 2.46 for male cohorts). Analysis using confirmed and non-confirmed cases showed similar results, except for autoimmune thyroiditis in females, OR: 1.45 (0.79 to 2.64) [20].
In the study by Angelo et al. the incidence of unsolicited AEFI reported within 30 days after administration was similar between HPV and Control groups (30.8% and 29.7%). The most frequently reported events within one year of administration were: psoriasis, Grave's disease, autoimmune thyroiditis and vasculitis, rheumatoid arthropathies and neuritis. The OR for each event showed no increased risk for women vaccinated with HPV vaccine [19].

## Discussion

The objective of this meta-analysis was to assess the onset of autoimmune conditions related to HPV vaccines. References were included if they reported a RCT of HPV vaccines, including a placebo control group and gave information regarding the onset of ADs [15-20].
We identified three studies reporting on bivalent HPV vaccines and three studies on quadrivalent vaccines. The total number of subjects included in the meta-analysis comprised 243,289 in the vaccine group and 248,820 in control groups (another vaccine or no vaccine).
Four of the six trials had a Jadad score of 3 or 4 indicating an adequate trial quality [17-20].
ADs were reported by all studies. However, none of the observed events were considered to be related to the use of HPV vaccine.
The results of the meta-analysis should be interpreted with caution due to the several limitations.
First the number of the included clinical trials meeting the inclusion criteria was limited; second due to the different geographical locations of the RCTs included in our study and the Higgins index obtained, we chose a random-effects model for the meta-analysis which further widens the confidence intervals. Differences in the

**Fig. 2.** Forest plot.

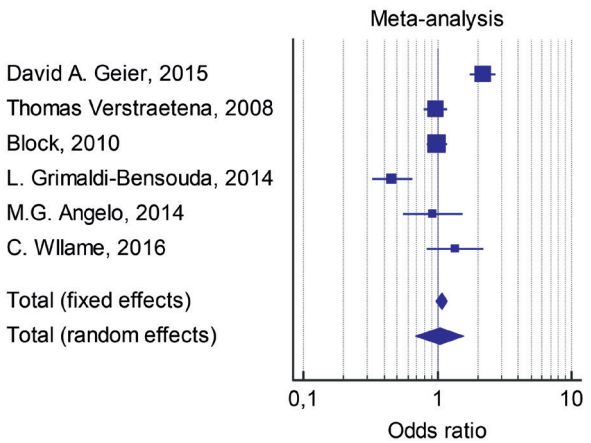
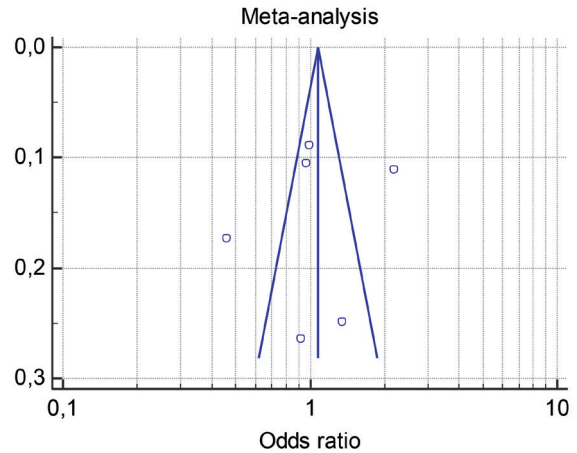



**Fig. 3.** Funnel plot.



reporting method of observed ADs and the different ADs reported also limit the results of the study.
Vaccine administration is usually safe and serious adverse events rare. In the past, the hypothesis of a correlation between the Hepatitis B vaccine and MS in adolescents, supported by reports of temporal association between vaccine shot and disease onset was sufficient to fuel controversies on the use of vaccine in subjects with other ADs [21-24]. This lesson about the effect of publications about possible links between ADs and a vaccine should thus be considered when the safety of a vaccine is debated; the risk of misinterpretation of association is particularly high when we consider autoimmune disease and the HPV vaccine, because this vaccine is recommended for young females (but also for males) in whom the incidence of autoimmune disease is high [25].
Therefore the role of pharmacovigilance surveillance remains of fundamental importance in allowing the scientific community to detect unknown or rare events possibly related to the vaccine.
A recent report highlighted the possible role of a genetic predisposition to vaccine-induced autoimmune disease [26]. The presence of genetic bases for adverse events has been described for several drugs; perhaps one of the most important being the HLA-B*57:01 for a nucleoside inhibitors of reverse transcriptase (abacavir) [27]. The identification of genetic bases for adverse events following vaccination should be actively investigated as this would provide a useful tool to prevent rare and serious diseases without impacting negatively on public confidence in immunisation programmes. Furthermore, many cases of ADs reported in the literature were sporadic cases appearing in articles such as "case reports". It is therefore necessary to clarify whether any relation exists between the administration of the vaccine and the onset of ADs (this would not seem to be so from our meta-analysis) or whether there is a mere coincidence in a subject destined to developed an ADs [28-30].
Today, concerns about vaccine safety have led some parents to decline recommended vaccination of their children, carrying to the spread of a phenomena called "vaccine hesitancy" and leading to the spread of diseases, as measles in Italy [31]. So study about the reassurance of vaccine safety remains critical for population health. In the literature, many reviews and meta-analysis analyzed the vaccine safety and one evidenced that there aren't association between some vaccines and AEFI as autism and leukemia, but show an association with some vaccines, such as intussusception after rotavirus vaccine or febrile seizure post MMR or MMRV vaccine [32-34].
These AEFI are extremely rare, many factors could be implicated and should be evaluated against the protective benefits provided by the vaccines.

## Conclusions

No major ADs were identified for bivalent and quadrivalent HPV vaccines. Therefore, further studies are needed, particularly with accurately defined and reported safety outcomes to better evaluate the risks of these vaccines. In future, we also aim to investigate the implications of HPV vaccines for the most commonly reported individual ADs.

## Acknowledgements

Funding sources: this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Conflict of interest statement

None declared.

## Authors' contributions

All authors carried out a systematic review to identify all scientific publications dealing with the correlation between vaccine anti-papillomavirus and new onset of autoimmune diseases. Screening was carried out independently by two authors (RS, CG). Any disagreement about eligibility between reviewers was resolved by a third author (VLF). The first two authors extracted data from included papers using a data extraction form reviewed by the other co-authors. Two independent reviewers (CG and RS) identified potentially relevant articles, collected the data and independently assessed the quality of individual studies included in the meta-analysis. GT and CG make data analysis.

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■ Received on July 30, 2018. Accepted on August 30, 2018.

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ORIGINAL ARTICLE

# Health and safety of pesticide applicators in a high income agricultural setting: a knowledge, attitude, practice, and toxicity study from North-Eastern Italy

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Keywords

Pesticides • Agricultural worker • Knowledge attitudes and practices (KAP) • Personal hygiene and sanitation • Personal protective equipment (PPE)

Summary

**Background**. *We assessed knowledge, attitudes and practices regarding pesticide handling and related health problems among pesticide applicators (PAs) from the Autonomous Province of Trento, Italy.*

**Methods**. *A cross-sectional questionnaire-based study was performed in spring 2016, involving 260 PAs. Logistic regression analyses were used to identify factors associated with a safer use of pesticides.*

**Results**. *The mean age of participants was 48.8 ± 13.2 years. 89.2% were males. Use of personal protective equipment (PPE) was diffuse, particularly gloves (92.7%), face mask (91.2%), and post-spraying personal hygiene practices were extensively applied. Overall, 43.5% had experienced pesticide-related symptoms especially in subjects misusing PPE, but also for avoiding hygienic procedures. Knowledge about pesticides was a significant predictor for frequency of symptoms.*

**Conclusions**. *As a better knowledge of pesticide-related risks was a significant predictor to reduce symptoms, our results stress that improving awareness and promoting safe use of pesticide may improve the health of PAs.*

## Introduction

Pesticides are known human and environmental toxicants, and are widely used throughout the world in order to assure crop protection against pests and guarantee high crop yields [1-4]. Even though several products have been banned due to their acute and chronic effects [5], pesticide applicators (PAs) are often not aware that also modern pesticides retain a significant toxicological profile, with a consequent global health burden [6-9]. Even though two-thirds of the 350,000 annual pesticide-related deaths occur in developing countries, figures remain of significant relevance also for high income countries [10, 11]. In Italy, for example, a total of around 2,500 occupational cases of acute pesticide intoxications were identified between 2005 and 2011, representing 5% of all poisonings [12].

Unsafe occupational exposure to these "poisons by design" has been associated with the lack of product knowledge and safety awareness among handlers [3, 13-16]. More specifically, available studies suggest that inappropriate practices and attitudes (i.e. use of banned substances, overspraying, lack of self-protection, incorrect storage, mishandling of pesticide containers, reuse of washed pesticide tanks as containers for food and drinking water) [2, 17-22], would be associated with significant knowledge and information gaps [1, 11, 13, 16, 23].

Even though educative interventions have been acknowledged as an appropriate approach for reducing occupational exposures in farmers [2-4, 24], a growing evidence suggests that applicants' behaviour may be quite more complex. First at all, subjects who are aware of the risks may still misuse pesticides to avoid a lower crop production [25], or may find the use of protective devices very uncomfortable (especially respirators, facemasks and overalls) because of the climate factors, such as high humidity and temperature [26]. Moreover, education may be not enough to bring about behaviour changes, being overwhelmed by personal experiences on pesticide side effects, social and shared interactions, in particular with retailers and resellers [11]. Finally, contextual and structural factors such as availability of appropriate materials and facilities (e.g. soap, water, gloves, showering facilities etc.), employers' and workers' commitment to occupational health and safety, likely influence the application of recommended protective behaviours [2-4, 11, 24, 25, 27].

Unfortunately, the majority of available researches regarding knowledge (i.e. the awareness of official recommendations regarding pesticide handling), attitudes (i.e. propensity towards preventive measures) and practices (i.e. actual prevalence of appropriate handling practices), or KAP, of pesticide applicators (PAs) has been performed in workers from highly or relatively deprived socioeconomic environments [1, 4, 11, 13, 20, 23, 28-30]. In order to as-

sess how inappropriate KAP do affect modern PAs from highly developed, and profitable farm settings, the present study was therefore carried out having the following objectives:

- assessment of the knowledge of farmers regarding the use of pesticides, their effects, their route of entry into the body;
- investigation of the determinants and predictors of poor/good knowledge;
- evaluation of the practices for the storage, preparation and disposal of pesticides;
- evaluation of field practices with pesticides in relation to the farmers' knowledge including use of personal protective equipment (PPE);
- determination of the prevalence of self-reported health symptoms associated with pesticide exposure and their relation to work practices.

## Materials and methods

### Subjects and settings

The present questionnaire-based cross sectional study included PAs from the Autonomous Province of Trento (APT). APT (537,416 inhabitants at 2015 census) is located in Italy's North East, covering a total area of 6,214 $km^2$. Its territory is overwhelmingly mountainous (70% over 1,000 m above sea level), and agricultural area only accounts for 22% of the total surface, but highly profitable cultivations (i.e. apples, vineyard) have significantly sustained the regional economic development. According to labour force statistics, the agricultural sector in APT directly accounts for around 20,000 employees (dependent and self-employed) in 11,958 agricultural enterprises, usually of small extent (89% are smaller than 5 hectares and 56% smaller than 1 hectare). These figures however do not include part-time employees, whose number may largely exceed full-time employees [31].
Despite local policies have extensively promoted the biological control of pests, between 2001 and 2012, a mean of 9.1 ± 0.6 kg/ha of pesticides has been used in the APT, significantly greater than in the rest of the country (6.1 ± 0.7 kg/ha). Among the pesticides more extensively used are in particular fungicides (1,893 T; 37.1 kg/ha), followed by insecticides (338 T; 10.6 kg/ha) and herbicides (111 T; 1.8 kg/ha) [32].

### Study participants

Sampling was performed by convenience. In APT, PAs must obtain professional certification in order to buy, sell, manage pesticides, irrespective of the volumes of the chemicals effectively used, or the frequency of their handling. Individuals participating to certification courses held between March and December 2016 were invited to participate (n = 366). Participants giving their preliminary consents shortly before the beginning of the courses received by hand a structured questionnaire that inquired their KAP regarding the use of pesticides. In order to avoid that the content of courses may influence participants regarding their answers, delivery and gathering of questionnaires was performed before the beginning of the courses.

### Instruments

A questionnaire based on the WHO field survey of Exposure to Pesticides Standard Protocol and similar studies [4, 28, 29, 33] was prepared and preliminary tested in 30 farm workers, who did not participate in the final study and completed the questionnaire at two different points in time in order to test-retest reliability of questionnaire items. A correlation coefficient was calculated to compare the two sets of responses: items having a coefficient > 0.80 were interpreted as consistent and, therefore, were included in the questionnaire used in this survey. All questions were self-reported and not externally validated and included the following items:

- Basic information about the interviewee, such as gender, age, educational level, and farming practices (crop types and yields, agrochemical products used etc.).
- The practice of pesticide application, including attitudes regarding the use of pesticides and PPE or clothes during preparation and application of pesticides; practice questions included: the wearing of PPE; following label instructions; smoking, eating, drinking water or chewing gum during application of pesticides; whether to have a water bath or not after application; and whether they complied with the safety period and concentration recommended.
- The level of awareness of the dangers of the pesticides, including knowledge of the acute and chronic toxicity of pesticides, effects of pesticides on human health, the route of pesticide entry into the human body. The responses were documented as "yes", "no" or "don't know". A cumulative knowledge score (KS) was calculated by awarding a score of "+ 1" for each "correct" response, whereas for "wrong" answer and for "don't' know" a score of "0" was given.
- Health impact of exposure to pesticides as self-reported symptoms associated with pesticide use; participants were asked to rate through a 5-point Likert scale (i.e. never, almost never, occasionally/sometimes, almost every time, every time) the frequency of 11 symptoms usually associated with pesticide use (i.e. headache; eyes itching; vision disturbances; shortness of breath; dizziness; nausea, vomiting, diarrhoea; salivation; skin rash/redness of skin; abdominal pain; disturbances of memory; tremor at rest); a cumulative symptom score (SyS) was then calculated by awarding a score of "+ 1" for a symptom frequency rated as "never", "+ 2" for "almost never", and so on.

### Ethics

Before they received the questionnaires, inquired subjects were informed that participation to the present survey was voluntary, and that the questionnaires would be gathered only in subjects expressing preliminary consent for study participation. Participants were guaranteed that they may withdraw from the survey in any time, by simply not delivering the questionnaire at the end of the course session,

and that all collected information would be handled anonymously and confidentially. As the questionnaire was strictly anonymous, it is implausible that individual participants could be identified based on the presented material, and ultimately this study caused no plausible harm or stigma to participating individuals. Moreover, as the final examiners of professional courses were totally blind regarding the status of inquired subjects (i.e. whether they had participated or not to the survey), it is also improbable that individual participants have felt forced to give their consent. As the study design assured an adequate protection of study participants, and neither include clinical data about patients nor configure itself as a clinical trial, competent Ethical Committee of the Provincial Agency for Health Services reputed a preliminary evaluation as not required.

### Data analysis

Two independent researchers, one of whom read the responses from each questionnaire while the other researcher reviewed the entered data, ensured the accuracy of data entry. The primary investigator examined unclear responses to determine the correct answer. Questionnaire lacking basic information about the interviewee were excluded from the study. Continuous variables were tested for normal distribution (D'Agostino & Pearson omnibus normality test): where the corresponding p value was < 0.10, normality distribution was assumed as rejected and variables were compared through Mann-Whitney or Kruskal-Wallis test for multiple independent samples. On the other hand, variables passing the normality check (D'Agostino & Pearson p value ≥ 0.10) were compared using the Student's t test or ANOVA, where appropriate.
Association of discrete variables with the presence of symptoms was initially assessed through chi-squared test. All variables whose association with self-symptoms was significant at univariate analysis, were then assessed by stepwise binary logistic regression analysis as adjusted OR with the respective 95% CI.
A linear regression analysis was also modelled in order to assess the relative influence of the KS, on the dependent variable SyS. The model included personal attitudes (i.e. information sources, reading of pesticide labels/instructions), and assessed practices (i.e. appropriate storage and disposal, appropriate use of PPE and personal hygiene practice) as covariates.
All regression analyses were controlled for age, sex and ethnicity (i.e. Italian born vs non-Italian born people) and performed on SPSS 24 (IBM Corp. Armonk, NY). Significance level was $p < 0.05$ for all calculations.

## Results

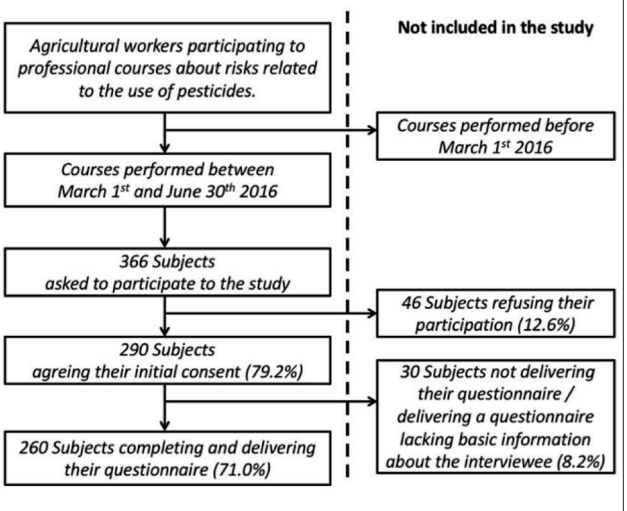
### Socio-demographics characteristics of the farmers participating to the study (Tab. I)

A total of 260 questionnaires were ultimately retrieved (Fig. 1), with a response rate of 71.0%, including 243 Italians (93.5%) and 17 subjects from a migration background (6.5%). The mean age of the participants was 48.8 ± 13.2 years, with PAs older than 50 years representing the 52.3% of the sample, that included 232 males (mean age 49.2 years ± 13.3) and 28 females (mean age 45.8 years ± 12.5, p = 0.195). Overall, 71 subjects (27.3%) were either current or past smokers. All the participants had completed at least the primary education (5 + 3 years), and 68.4% of them had achieved secondary education level (13 years) or higher. Majority of respondents performed pesticide handling as professional farmers (64.2%), either self-employed (52.7%) or salaried farm workers (11.5%), whereas 35.8% of participants self-styled as "hobby farmers". Main crops were fruits or vegetables (75.0%), followed by flowers (25 subjects, 9.6%), and mixed cultures (12.7%).

**Tab. I.** Demographics of 260 pesticide applicators from the Autonomous Province of Trento participating to the study (2016).

| Category | Variables | N | % |
|---|---|---|---|
| Gender | Male | 232 | 89.2 |
| | Female | 28 | 10.8 |
| Origin | Italian born people | 243 | 93.5 |
| | Foreign born people | 17 | 6.5 |
| Smoking status | Current smoker | 71 | 27.3 |
| | No smoker | 189 | 72.7 |
| Age | ≤ 30 | 28 | 10.8 |
| | 30-39 | 33 | 12.7 |
| | 40-49 | 63 | 24.2 |
| | ≥ 50 | 136 | 52.3 |
| Occupational status | Farmer (owner) | 137 | 52.7 |
| | Hobby farmer | 93 | 35.8 |
| | Farmer (employee) | 30 | 11.5 |
| Education level | University or more | 31 | 11.9 |
| | High school or greater | 147 | 56.5 |
| | Primary/Secondary school | 82 | 31.5 |
| Type of crop grown | Fruits or Vegetables | 202 | 77.7 |
| | Flowers | 25 | 9.6 |
| | Mixed | 33 | 12.7 |
| Type of agricultural fields | Open fields | 224 | 86.2 |
| | Closed fields | 11 | 4.2 |
| | Both open and closed fields | 25 | 9.6 |

**Fig. 1.** Flow-chart explaining the selection of the study sample.



### Attitudes, practices and behaviours (Tab. II)

Professional handling of pesticides for at least 5 years was reported by 74.6% of participants, with around a third (n = 98, 37.7%) for over 20 years, and the majority of the respondents reported to handle pesticides at least 3 times per month during the crop season (63.5%). Pesticide labels and instructions were regularly read by 92.7% of participants, whereas 5.0% of them omitted their assessment because the information was "too complicated". More specifically, the most frequently referred information source was represented by pesticide resellers (33.8%), followed by personal experience (23.1%) and professional courses (17.7%), while the least reported was represented by health profession-

Tab. II. Attitudes, practices and personal behaviours related to the use of pesticides in 260 pesticide applicators from the Autonomous Province of Trento (2016).

| Variables | | N | % |
|---|---|---|---|
| Professional courses attended about the safe use of pesticides | None in the last 5 years | 213 | 81.9 |
| | At least one in last 5 years | 47 | 18.1 |
| Days, per month, working with pesticides | 1-2 days | 92 | 35.4 |
| | 3-10 days | 132 | 50.8 |
| | 11 days or more | 36 | 13.8 |
| Years of pesticide use | 1-5 years | 66 | 25.4 |
| | 6-10 years | 47 | 18.1 |
| | 11-20 years | 49 | 18.8 |
| | > 20 years | 98 | 37.7 |
| Main information sources about pesticides | Resellers | 88 | 33.8 |
| | Personal experience | 60 | 23.1 |
| | Professional courses | 46 | 17.6 |
| | Conventional media | 22 | 8.5 |
| | New media | 19 | 7.3 |
| | Friends/Relatives | 16 | 6.2 |
| | Health professional | 9 | 3.5 |
| Pesticide label or instructions | Reading, regularly | 241 | 92.7 |
| | Not reading | 19 | 7.3 |
| Storage[1] | Home | 26 | 10.0 |
| | Specific store nearby home | 112 | 43.1 |
| | Animal house | 7 | 2.7 |
| | Farm site | 124 | 47.7 |
| Disposal of leftover pesticides[1] | Specific disposal | 213 | 81.9 |
| | Storing for reuse | 49 | 18.8 |
| | Burying | - | - |
| | Pour in the field | 24 | 9.2 |
| Disposal of empty containers[1] | Specific disposal | 243 | 93.5 |
| | Washing and reusing | 13 | 5.0 |
| | Burying | - | - |
| | Leave in the field | 3 | 1.2 |
| | I prefer not explain it | 1 | 0.4 |
| Self-referred use of Personal Protective Equipments, and personal practices performed during spray[1] | Face mask | 237 | 91.2 |
| | Gloves | 241 | 92.7 |
| | Eye mask | 180 | 69.2 |
| | Specific shoes | 195 | 75.0 |
| | Specific clothes | 198 | 76.2 |
| | Impermeable clothes | 169 | 65.0 |
| | Long sleeve clothes | 219 | 84.2 |
| | Hat / Hood | 210 | 80.8 |
| | Consume food and/or water | 18 | 6.9 |
| | Drink alcohol | 10 | 3.8 |
| | Smoking | 20 | 7.7 |
| | Chewing gum | 10 | 3.8 |
| Self-referred personal practices of the participants after spray[1] | Replace/clean face mask and/or filters | 204 | 78.5 |
| | Replace/clean gloves | 199 | 76.5 |
| | Wait at least 24 h to re-enter | 258 | 99.2 |
| | Change clothes | 219 | 84.2 |
| | Clean/wash clothes | 210 | 80.8 |
| | Wash hands | 246 | 94.6 |
| | Take a shower/bath | 224 | 86.2 |
| | Consume food and/or water | 199 | 76.5 |
| | Drink alcohol | 243 | 93.5 |
| | Smoking | 212 | 81.5 |
| | Chewing gum | 232 | 89.2 |

[1]: as multiple answers were allowed, total sum may exceed 100%.

als such as occupational physician (3.5%). In addition, 47 PAs (18.1%) claimed that they had participated in at least one professional course (not including the present one) in the previous 5 years.

The majority of participants (90.8%) claimed that there was a special site for pesticide storage, either nearby home or in the farm, and only 26 individuals (10.0%) reported storing these products inside their house. The empty pesticide containers were returned to the specific disposal program by 93.5% of respondents, whereas 13 (5.0%) washed and reused the containers. Similarly, majority of participants disposed leftover pesticides through specific programs (81.9%), 18.8% stored them for reuse and 9.2% simply poured them on the fields. No one reported to have buried or burned the containers or leftover pesticides, but 1 participant (0.4%) declared that he preferred to not share information about the management of containers.

Focusing on the use of PPE during the disposal of pesticides, the majority of participants regularly wore specific gloves (92.7%), a face mask (91.2%), long sleeve clothes (84.3%), and a hat or a hood (80.8%). Similarly, a high share of respondents mentioned not drinking and/or eating (96.9%), not smoking (92.3%), and not chewing gum (96.2%). After pesticide handling, the majority of respondents reported to regularly wash the hands (94.6%), taking a shower or a water bath (86.2%), managing the face mask or the filters (88.5%), changing (84.2%) or cleaning/washing the clothes (80.8%), and replacing/cleaning the gloves (76.5%). Eventually, 76.5% of PAs reported that they did not consume food and/or drink water after the pesticide dispersal, whereas 18.5% referred to regularly smoke and 10.8% chewed gum.

### Knowledge of farmers about health effects of pesticides

The potential range of KS was 0-33, and we assessed a mean of 23.4 ± 5.7 (actual range: 11 to 33). Focusing on the single statements (Tab. III), the majority of the participants were aware that pesticide may have health effects for applicators (85.0%) and nearby residents (76.5%), that their use may cause potentially lethal intoxications (90.0%) and long term effects (71.2%), and that pesticides may similarly affect surface waters (99.2%), pollinating insects (96.9%) and livestock (96.2%), ultimately involving the soil fertility (78.1%). Moreover, farmers had considerable high knowledge that pesticides may accumulate in groundwater and surface waters (96.5%), in the soil (92.3%), and eventually in fruits/vegetables (91.9%). All participants acknowledged the skin as a main route of entry of pesticides in the human body, whereas 99.6% and 93.8% claimed nose and mouth as a possible route of entry, respectively. Actually, majority of the farmers were aware that pesticides should be handled by using PPE only (93.8%), but almost a third of the sample (27.3%) believed that the human body may ultimately develop some form of resistance against the used pesticides.

The best known consequences of pesticide exposure were itching eyes (80.4%), followed by skin rash or redness of the skin (73.8%), visual disturbances (73.5%), shortness of the breath (71.2%), nauseas, vomiting, and diarrhoea (67.7%). Around 60% of the participants identified neoplasia as a pesticide-related health effect (58.5%), and nearly 40% of the farmers identified the risk for neurological disorders such as Parkinson's or Alzheimer's disease (37.7%). Finally, the least known health effects were stillbirth / abortion (29.6%), congenital malformations (27.7%) and forgetfulness (15.8%).

KS significantly increased with education level ($p < 0.001$), and graduated PAs had mean scores (26.3 ± 4.6) significantly higher than subjects referring high school (24.1 ± 5.5) and primary/secondary school (21.1 ± 5.7) degree. KS conversely decreased with the years of pesticide use, being higher in subject referring 1 to 5 years of experience (25.9 ± 4.6), and lower in participants handling pesticides for 20 or more years (22.3 ± 5.9, $p = 0.001$) (Tab. IV).

A better KS was then identified in subjects who had attended at least one professional course in the previous 5 years (23.7 ± 5.7 vs 21.7 ± 5.6, $p = 0.030$) and growing flowers (23.6 ± 6.0) rather than fruits or vegetables (23.6 ± 6.0) or even mixed cultures (22.7 ± 5.7, $p = 0.001$). Focusing on information sources, a better score was identified in subjects relying on health professionals (26.9 ± 1.6) and professional courses (25.6 ± 4.4), whereas PAs referring new media (22.5 ± 8.1), resellers (22.5 ± 5.5), and in particular friends/relatives (20.1 ± 4.7) had the lowest ones.

### Self-reported toxicity symptoms and their association with personal characteristics/behaviour

A total of 113 participants (43.5%) self-reported every time or almost every time they handled pesticides at least one of the listed symptoms, with a mean SyS of 15.0 ± 6.3 (potential range of 11 to 55; actual range 11 to 52). As shown in Figure 2, itching of the eyes (72, 27.7%), vision disturbances (63, 24.2%), headache (53, 20.4%) were the most common ones, and forgetfulness (12, 4.6%), abdominal pain, resting tremor (both symptoms: 11, 4.2%) and dizziness (10, 3.8%) the least common.

Prevalence of toxicity symptoms was not significantly affected by main demographic variables, including sex, age, education level and occupational status (Tab. V). Focusing on work-related practices, only workers having a pesticide use history of 20 years or more referred a significantly increased prevalence of symptoms, whereas frequency of pesticide application was unrelated with the prevalence of symptoms.

A greater prevalence of toxicity symptoms was referred by subjects not wearing a face mask, specific clothes, gloves, impermeable shoes, and eye mask during pesticide dispersal. Similarly, farmers not washing their hands, not replacing working clothes and gloves, as well as drinking alcohol, chewing gum, and smoking after the spray had a significant association with self-referral of symptoms (Tab. VI).

**Tab. III.** Knowledge of 260 pesticide applicators from the Autonomous Province of Trento about health effects of pesticides, including symptoms potentially related to pesticide use.

| | Correct | Incorrect | Don't know |
|---|---|---|---|
| **Knowledge regarding pesticide use** | | | |
| Pesticides are deprived of health effects for applicators | 221 (85.0%) | 24 (9.2%) | 15 (5.8%) |
| Pesticides enter into the body through the skin | 260 (100.0%) | - | - |
| Pesticides enter into the body through the mouth | 244 (93.8%) | 9 (3.5%) | 7 (2.7%) |
| Pesticides enter into the body through the nose | 259 (99.6%) | 1 (0.4%) | - |
| Pesticides affect livestock | 250 (96.2%) | 1 (0.4%) | 9 (3.5%) |
| Pesticides affect pollinating insects (e.g. bees) | 252 (96.9%) | 5 (1.9%) | 3 (1.2%) |
| Pesticides affect surface water | 258 (99.2%) | - | 2 (0.8%) |
| Pesticides affect soil fertility | 203 (78.1%) | 36 (13.8%) | 21 (8.1%) |
| Pesticides induce only short-term effects | 185 (71.2%) | 68 (26.2%) | 7 (2.7%) |
| Pesticides accumulate in the soil | 240 (92.3%) | 11 (4.2%) | 9 (3.5%) |
| Pesticides affect only applicators, not nearby residents | 199 (76.5%) | 55 (21.2%) | 6 (2.3%) |
| Pesticides accumulate in groundwater and surface waters | 251 (96.5%) | 9 (3.5%) | - |
| Pesticides accumulate in fruits and vegetables | 239 (91.9%) | 14 (5.4%) | 7 (2.7%) |
| Some pesticides have been banned | 226 (86.9%) | 18 (6.9%) | 16 (6.2%) |
| Some pesticides may cause potentially lethal intoxications | 234 (90.0%) | 12 (4.6%) | 14 (5.4%) |
| Human body develop resistance against pesticides | 126 (48.5%) | 71 (27.3%) | 63 (24.2%) |
| Pesticides should be handled only using PPE | 244 (93.8%) | 13 (5.0%) | 3 (1.2%) |
| Pesticides affects only children and elders | 247 (95.0%) | 6 (2.3%) | 7 (2.7%) |
| **Health effects associated with pesticide use** | | | |
| Headache | 167 (64.2%) | 54 (20.8%) | 39 (15.0%) |
| Eyes itching | 209 (80.4%) | 32 (12.3%) | 19 (7.3%) |
| Vision disturbance | 191 (73.5%) | 40 (15.4%) | 29 (11.2%) |
| Shortness of breath | 185 (71.2%) | 45 (17.3%) | 30 (11.5%) |
| Dizziness | 107 (41.2%) | 73 (28.1%) | 80 (30.8%) |
| Nausea, vomiting, diarrhoea | 176 (67.7%) | 53 (20.4%) | 31 (11.9%) |
| Salivation | 105 (40.4%) | 64 (24.6%) | 91 (35.0%) |
| Skin rash/redness of skin | 192 (73.8%) | 43 (16.5%) | 25 (9.6%) |
| Abdominal pain | 83 (31.9%) | 82 (31.5%) | 95 (36.5%) |
| Forgetfulness | 41 (15.8%) | 115 (44.2%) | 104 (40.0%) |
| Congenital malformations | 72 (27.7%) | 85 (32.7%) | 103 (39.6%) |
| Fever/shivering | 80 (30.8%) | 77 (29.6%) | 103 (39.6%) |
| Neoplasia | 152 (58.5%) | 50 (19.2%) | 58 (22.3%) |
| Stillbirth/abortion | 77 (29.6%) | 65 (25.0%) | 118 (45.4%) |
| Neurological disorders (e.g. Parkinson's, Alzheimer's disease) | 98 (37.7%) | 61 (23.5%) | 101 (38.8%) |

### Regression analysis

In multivariate analysis, after adjustments for sex, age, cultivation type, years of experiences, previous frequency to formation courses, all aforementioned practices and behaviours were confirmed as significantly associated with a positive status for toxicity symptoms. In particular, not washing hands (OR 36.343 95% CI 4.206-313.0), drinking alcohol (OR 20.718 95% CI 4.154-103.4), and chewing gum (OR 7.054 95% CI 2.635-18.884) after spraying, and the inappropriate use of PPE such as face mask (OR 7.849 95% CI 2.610-23.604), gloves (OR 5.972 95% CI 1.979-18.023) and shoes (OR 3.822 95% CI 1.988-7.349) were associated with higher prevalence of self-reported symptoms (Tab VI).
In linear regression analysis, SyS found a statistically significant predictor in KS (B coefficient = – 0.204, 95% CI – 0.325 to – 0.082, $p < 0.001$), i.e. farmers having a better awareness of the risk related to pesticide handling had a lower frequency of pesticide related symptoms.

## Discussion

Agricultural workers and PAs are usually described as affected by an extensive lack of knowledge about the risks associated with pesticide handling [1, 6, 20, 34]. As the majority of the available surveys were performed in developing countries, or included workers from relatively or even highly deprived socioeconomic environments, the consistency of these reports with high-income countries, with a higher ratio of highly profitable cultivations, and where the use of pesticides is strictly regulated, might therefore be disputed [1, 4, 11, 13, 20, 23, 28-30]. Our results suggest that PAs from a high-developed agricultural system may exhibit satisfying knowledge of health and environmental effects of pesticides, and are

**Tab. IV.** Knowledge score by demographic factors and main attitude towards the use of pesticides in 260 pesticide applicators from the Autonomous Province of Trento.

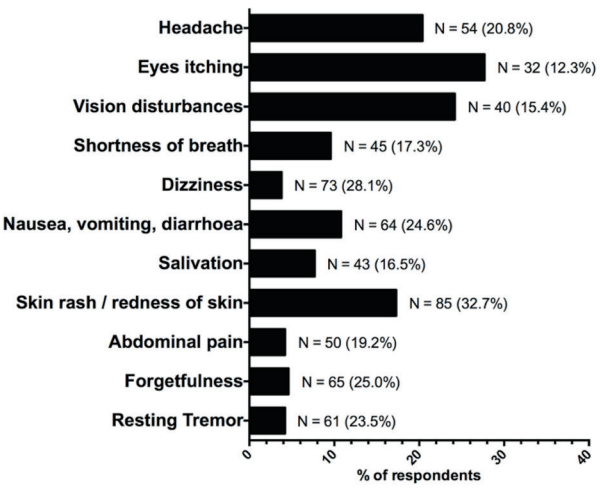
| Category | Variables | Knowledge score | P value |
|---|---|---|---|
| Gender | Male<br>Female | 23.4 ± 5.7<br>23.5 ± 5.8 | 0.911 |
| Origin | Italian born people<br>Foreign born people | 23.5 ± 5.7<br>22.4 ± 6.3 | 0.466 |
| Smoking status | Current smoker<br>No smoker | 22.7 ± 4.6<br>23.6 ± 6.1 | 0.189 |
| Age | ≤ 30<br>30-39<br>40-49<br>≥ 50 | 24.1 ± 5.1<br>24.6 ± 6.0<br>23.0 ± 5.9<br>23.2 ± 5.7 | 0.512 |
| Occupational status | Farmer (owner)<br>Hobby farmer<br>Farmer (employee) | 23.8 ± 6.0<br>22.8 ± 5.3<br>23.8 ± 5.6 | 0.424 |
| Education level | University or more<br>High school or greater<br>Primary/Secondary school | 26.3 ± 4.6<br>24.1 ± 5.5<br>21.1 ± 5.7 | < 0.001 |
| Type of crop grown | Fruits or Vegetables<br>Flowers<br>Mixed | 23.6 ± 6.0<br>26.5 ± 4.0<br>22.7 ± 5.7 | 0.001 |
| Type of agricultural fields | Open fields<br>Closed fields<br>Both open e closed fields | 23.4 ± 5.7<br>21.4 ± 4.7<br>24.2 ± 6.0 | 0.391 |
| Professional courses | None in the last 5 years<br>At least one in last 5 years | 21.7 ± 5.6<br>23.7 ± 5.7 | 0.030 |
| Days, per month, working with pesticides | 1-2 days<br>3-10 days<br>11 days or more | 21.1 ± 6.4<br>23.6 ± 5.2<br>23.3 ± 5.9 | 0.823 |
| Years of pesticide use | 1-5 years<br>6-10 years<br>11-20 years<br>> 20 years | 25.9 ± 4.6<br>24.1 ± 5.7<br>22.3 ± 5.9<br>22.1 ± 5.7 | 0.001 |
| Main information sources about pesticides | Resellers<br>Personal experience<br>Professional courses<br>Conventional media<br>New media<br>Friends/Relatives<br>Health professional | 22.4 ± 5.5<br>23.7 ± 5.8<br>25.6 ± 4.4<br>23.5 ± 5.4<br>22.5 ± 8.1<br>20.1 ± 4.7<br>26.9 ± 1.6 | 0.004 |

presumptively related with some specificities of the studied population.

First at all, mean age of participants was relatively higher than that in previous studies, with 52.3% of them aged more than 50 years, but such figures were not unexpected as epidemiological studies clearly indicate that in developed countries agricultural workers (AWs) are significantly older than most other occupational groups [35-37].

Second, a high share of PAs reported a formal education > 13 years [29, 34, 38, 39]: in this regard, it should be stressed that in APT a significant number of farmers perform agricultural activities as a collateral and/or part-time job, eventually reverting to agricultural activities after their retirement [31]. Even though older farmers usually show significant knowledge gaps, and also in our study KS was significantly decreased in higher age groups, education level has been repetitively described as a main determinant for the misuse of pesticides [1, 6]. More specifically, subjects of lower education level apparently perceive lower risks and higher benefits from using pesticides, potentially leading them to incorrect behaviour [1, 6]. In particular, lower education is associated with inappropriate reading of pesticides labels, hazard warnings and provided instructions [20, 34], as they are perceived too technical and not understandable [25]. Third, as a consequence of the convenience sampling we performed, not only all the participants actually handled pesticides, but around 75% of them had managed pesticides for at least 5 years.

**Fig. 2.** Self-reported symptoms complained "every time" or "almost every time" by 260 pesticide applicators from the Autonomous Province of Trento after pesticide handling.




Not coincidentally, around 92.5% of the sample referred the regular reading of pesticide labels and instructions, and nearly 80% recalled adequate and reliable sources of information, such as resellers, professional courses, and also health professionals [4]. In effect, even though only 18.1% of the study participants referred to have attended at least a professional course about the safe use of pesticides in the previous 5 years, participants referring health professionals and specific professional courses as main information sources had the better KS of this survey. In this regard, oral communication with retailers was associated with more ambiguous results. On the one hand, it played a dominant role in learning about pesticides and their functions, but the resulting level of knowledge was apparently unsatisfying, ultimately emphasizing the need for specific education and supervision programmes for pesticide resellers [7].

Focusing on the referred practices, proper methods of storing pesticide was diffuse [8, 34, 40, 41], with most of participants referring a specific storage site either nearby home or in the farm site. Moreover, the majority of the participant referred a specific disposal of leftover pesticides and empty containers, as the latter were washed and reused only by 5.0% of the participants.

**Tab. V.** Prevalence of the self-reporting of one or more symptoms related with pesticide exposure among 260 farmers, broken down by demographics and attitude and pesticide-related behaviors. Odds ratios (OR) with their respective 95% confidence intervals were obtained through bivariate analysis.

| Variables | | Self-referred symptoms (any) | P value | OR (95%CI) |
|---|---|---|---|---|
| Gender | Male<br>Female | 99 (42.7%)<br>14 (50.0%) | 0.591 | - |
| Origin | Italians<br>Migration background | 106 (43.6%)<br>7 (41.2%) | 0.844 | - |
| Smoking status | Previous/Current smoker<br>Never smoker | 32 (45.1%)<br>81 (42.9%) | 0.784 | - |
| Age (years) | ≤ 30<br>30-39<br>40-49<br>≥ 50 | 9 (32.1%)<br>10 (30.3%)<br>33 (52.4%)<br>61 (44.9%) | 0.115 | - |
| Occupational status | Farmer (owner)<br>Hobby farmer<br>Farmer (employee) | 64 (46.7%)<br>36 (38.7%)<br>13 (43.3%) | 0.486 | - |
| Education level | University or higher<br>High school or higher<br>Primary/Secondary school | 12 (38.7%)<br>70 (47.6%)<br>31 (37.8%) | 0.303 | - |
| Type of crop grown | Fruits or Vegetables<br>Flowers<br>Mixed | 80 (39.6%)<br>13 (52.0%)<br>20 (60.6%) | 0.052 | - |
| Professional courses | None in the last 5 years<br>At least one in last 5 years | 98 (46.0%)<br>15 (31.9%) | 0.077 | - |
| Days, per month, working with pesticides | 1-2<br>3-10<br>11 or more | 37 (40.2%)<br>66 (50.0%)<br>10 (30.3%) | 0.083 | - |
| Years of pesticide use | 1-5<br>6-10<br>11-20<br>> 20 | 18 (27.3%)<br>19 (40.4%)<br>20 (40.8%)<br>56 (57.1%) | 0.002 | REF<br>1.810 (0.817-4.009)<br>1.839 (0.838-4.037)<br>3.556 (1.813-6.972) |
| Main information sources about pesticides | Resellers<br>Personal experience<br>Professional courses<br>Conventional media<br>New media<br>Friends/Relatives<br>Health professional | 40 (45.5%)<br>34 (56.7%)<br>18 (39.1%)<br>4 (18.2%)<br>7 (36.8%)<br>5 (31.3%)<br>5 (55.6%) | 0.055 | - |
| Pesticide label or instructions | Reading, regularly<br>Not reading | 104 (43.2%)<br>9 (47.4%) | 0.721 | - |
| Storage site of pesticides | Home<br>Specific store nearby home<br>Animal house<br>Farm site | 13 (50.0%)<br>46 (51.1%)<br>4 (57.1%)<br>56 (45.2%) | 0.724 | - |
| Disposal of leftover pesticides | Specific disposal<br>Storing for reuse<br>Pour in the field | 91 (42.7%)<br>22 (44.9%)<br>15 (62.5%) | 0.182 | - |
| Disposal of empty containers | Specific disposal<br>Washing and reusing<br>Leave in the field | 106 (43.6%)<br>6 (46.2%)<br>3 (100.0%) | 0.147 | - |

This is of particular interest not only for occupational health and safety, but also in broader terms, as storing pesticides at home or in inappropriate working environment can easily contaminate drinking water and food, ultimately threatening the health of other non-professionally exposed family members, whereas the disposal of the empty containers in the field or by throwing them near or into local waste containers has been reported as a major public health problem in a number of studies [1, 25, 29, 34, 38, 42]. Unsurprisingly, not only storing pesticides at home was associated with a lower KS, but also an appropriate storage and disposal were consistently associated with better scores.

Analysis of personal practices identified a more ambiguous pattern. On the one hand, available evidence suggests a general acknowledgement that the use of appropriate PPE (i.e. long-sleeved shirts, impermeable working clothes, work boots, gloves and a hat/hood) at spraying significantly decreases the probability of poisoning in pesticide handlers [8, 43]. On the other hand, personal hygiene measures such as washing hands, changing clothes, showering, and washing work clothes from

**Tab. VI.** Selected practices during and after spraying of 260 pesticide applicators from the Autonomous Province of Trento (2016), and their correlation with the presence/absence of symptoms. Adjusted Odds Ratio (OR) were calculated through regression analysis.

| Practices | Use | Symptoms | | P value | OR | 95% CI |
|---|---|---|---|---|---|---|
| | | Yes (113, 43.5%) | No (147, 56.5%) | | | (LL-UL) |
| **At spraying** | | | | | | |
| Wear: face mask | No<br>Yes | 18, 15.9%<br>95, 84.1% | 5, 3.4%<br>142, 96.6% | 0.026 | 7.849 | 2.610-23.6 |
| Wear: gloves | No<br>Yes | 13, 11.5%<br>100, 88.5% | 6, 4.1%<br>141, 95.9% | 0.017 | 5.972 | 1.979-18.0 |
| Wear: eye mask | No<br>Yes | 45, 39.8%<br>68, 60.2% | 35, 23.8%<br>112, 76.2% | 0.033 | 2.965 | 1.616-5.443 |
| Wear: specific shoes | No<br>Yes | 42, 37.2%<br>71, 62.8% | 23, 15.6%<br>124, 84.4% | 0.010 | 3.822 | 1.988-7.349 |
| Wear: specific clothes | No<br>Yes | 26, 23.0%<br>87, 77.0% | 36, 24.5%<br>111, 75.5% | 0.541 | - | - |
| Wear: impermeable clothes | No<br>Yes | 51, 45.1%<br>62, 54.9% | 40, 27.2%<br>107, 72.8% | 0.005 | 2.284 | 1.285-4.060 |
| Wear: long sleeve clothes | No<br>Yes | 20, 17.7%<br>93, 82.3% | 21, 14.3%<br>126, 85.7% | 0.170 | - | - |
| Wear: hat/hood | No<br>Yes | 19, 16.8%<br>94, 83.2% | 31, 21.1%<br>116, 78.9% | 0.738 | - | - |
| Consume food and/or water | Yes<br>No | 8, 7.1%<br>105, 92.9% | 10, 6.8%<br>137, 93.2% | 0.354 | - | - |
| Drink alcohol | Yes<br>No | 5, 4.4%<br>108, 95.6% | 5, 3.4%<br>142, 96.6% | 0.002 | - | - |
| Smoking | Yes<br>No | 10, 8.8%<br>103, 91.2% | 10, 6.8%<br>137, 93.2% | 0.250 | - | - |
| Chewing gum | Yes<br>No | 5, 4.4%<br>108, 95.6% | 5, 3.4%<br>142, 96.6% | 0.006 | - | - |
| **After spraying** | | | | | | |
| Replace/clean face mask and/or filters | No<br>Yes | 36, 31.9%<br>77, 68.1% | 20, 13.6%<br>127, 86.4% | 0.040 | 3.504 | 1.781-6.893 |
| Replace/clean gloves | No<br>Yes | 34, 30.1%<br>79, 69.9% | 27, 18.4%<br>120, 81.6% | 0.006 | 2.057 | 1.100-3.845 |
| Wait at least 24 h to re-enter | No<br>Yes | 2, 1.8%<br>111, 98.2% | 0<br>147, 100% | 0.042 | - | - |
| Change clothes | No<br>Yes | 28, 24.8%<br>85, 75.2% | 13, 8.8%<br>134, 91.2% | 0.003 | 4.629 | 2.022-10.6 |
| Clean/wash clothes | No<br>Yes | 24, 21.2%<br>89, 78.8% | 26, 17.7%<br>121, 82.3% | 0.008 | - | - |
| Wash hands | No<br>Yes | 13, 11.5%<br>100, 88.5% | 1, 0.7%<br>146, 99.3% | 0.001 | 36.343 | 4.206-313.0 |
| Take a shower/bath | No<br>Yes | 17, 15.0%<br>96, 85.0% | 19, 12.9%<br>128, 87.1% | 0.030 | - | - |
| Consume food and/or water | Yes<br>No | 27, 23.9%<br>86, 76.1% | 34, 23.1%<br>113, 76.9% | 0.135 | - | - |
| Drink alcohol | Yes<br>No | 15, 13.3%<br>98, 86.7% | 2, 1.4%<br>145, 98.6% | 0.001 | 20.718 | 4.154-103.4 |
| Smoking | Yes<br>No | 27, 23.9%<br>86, 76.1% | 21, 14.3%<br>126, 85.7% | < 0.001 | 2.358 | 1.186-4.688 |
| Chewing gum | Yes<br>No | 21, 18.6%<br>92, 81.4% | 7, 4.8%<br>140, 95.2% | 0.005 | 7.054 | 2.635-18.9 |

household laundry immediately after work have been also described as efficient in order to avoid poisoning after pesticide application [44], but are more inconsistently applied, and frequently neglected [40]. In facts, several studies have found detectable levels of pesticide residues on farm workers' work boots, clothes etc., suggesting a significant household contamination from inappropriate practices of personal hygiene measures [8, 41]. Not coincidentally, in our survey subjects correctly applying personal hygiene measure, in particular post-spraying, were associated with better KS, whereas it was unrelated with practices involving PPE.

These results may found several explanations [4, 29, 45]. First at all, the correlation between knowledge and practices may be not so straight as previously supposed. Available studies suggests that farmers may actually ignore appropriate preventive measure notwithstanding an appropriate awareness of related risks, not only in devel-

oping countries, because of factors other than knowledge of pesticide health effects [4, 25, 28, 38, 46]. For example, unavailability and/or inappropriate handling of PPE may be easily recognized and fined by work inspectors, and therefore the use of some PPE may be perceived by the worker more as a regulatory requirement rather than as a safety measure, ultimately operating the equipment without any understanding of its rationale [23]. Again, as climate scenarios project an increase in global mean temperature and in the frequency and intensity of heat waves over most areas around the world in the near future [47], rigorous usage of PPE becomes ever more difficult, especially in an increasingly older group of workers. Finally, we cannot rule out that the use of PPE may have been perceived by participants as the "socially appropriated" behaviour (i.e. social desirability bias), with our results ultimately overstating their actual use [48].

The prevalence of symptoms potentially related to pesticide intoxication may be interpreted as an outcome of knowledge, attitudes and practices of farmers regarding pesticide handling and personal protective measures, including both the use of PPE and personal hygiene practices [2-4, 11, 13, 20, 21, 23, 25, 28, 29, 45, 49, 50]. In our sample, prevalence of symptoms was relatively high (43.5%), and linear regression analysis identified KS as a significant predictor for their frequency. Consistently with previous reports, higher rates toxicity-related symptoms were also identified in subjects having a lower education level, and referring higher prevalence of inappropriate attitudes and practices. In particular, participants denying the use of face mask (adjOR 7.849, 95% CI 2.610-23.6), gloves (adjOR 5.972, 95% CI 1.979-18.0), eye mask (adjOR 2.965, 95% CI 1.616-5.433), specific shoes and impermeable clothes (adjOR 3.822, 95% CI 1.988-7.349 and 2.284, 95% CI 1.285-4.060, respectively) at spraying, and not replacing or cleaning respiratory tract PPE (adjOR 3.504, 95% CI 1.781-6.893), gloves (adjOR 2.057, 95% CI 1.100-3.845), clothes (adjOR 4.629, 95% CI 2.022-10.6), or simply not washing their hands (adjOR 36.343 95% CI 4.206-313.0) after spraying referred a significantly higher prevalence of complaints. Also the avoiding of simple post-spraying personal hygiene practices such as not smoking (adjOR 2.358, 95% CI 1.186-4.688) and not chewing gum (adjOR 7.054, 95% CI 2.635-18.9) was significantly associated with increased risk for pesticide related symptoms. Despite relatively few participants reported to drink alcohol immediately after spraying, the ratio of complaints was relatively high (15/17 vs 98/145; adjOR 20.718, 95% CI 4.154-103.4), and may be explained as a function of the high consumption of fungicides such as dithiocarbamate in the APT [31, 32].

Dithiocarbamate induced alcohol intolerance is actually well known, and the inhibitory effects on enzymes is only slowly reversible, ultimately increasing the risk for subjects performing repetitive treatments [1-8].

On the contrary, PAs relying on conventional media as main information source referred a significantly lower prevalence of intoxication symptoms, and we may tentatively explain these result as the assessing of an attitude towards the search for updated information rather as the effect of the information by itself, ultimately identifying subjects more proactive regarding the use of appropriate personal protective practices.

Collectively, these outcomes are somehow coherent with more recent studies suggesting a complex correlation between knowledge and practices in pesticide applicators. PAs may be aware that pesticides are harmful, still not applying appropriate measures to avoid and/or reduce exposure [25], not only because of misbeliefs or even false beliefs regarding pathways of exposure and specific health effects, but because of personal interest [2-4, 11, 13, 20, 21, 23, 25, 28, 29, 45, 49, 50].

However, it should be stressed that studies inquiring symptoms in farmers and PAs handling pesticides are limited in number, and their reliability is usually limited by the usual self-assessment of symptoms, frequently identified as a weak indicator for pesticide poisoning for several reasons, and also our study shares these limitations [2-4, 11, 13, 20, 21, 23, 25, 28, 29, 45, 49, 50]. On the one hand, as pesticides are frequently recognised "poisons by design", participants may potentially overstate their complaints during and after spraying, reporting them in terms of "common-sense" rather than as a consequence of their previous occupational experience [15, 44, 50].

On the other hand, most of pesticide-related symptoms are very common disorders. Headache, eye discomfort, visual disturbances, together with nausea/vomiting, and skin disorders have been frequently reported after pesticide use, and these conditions are not only very common in the general population, but are also potentially correlated with other occupational exposures such as pollens, dusts and UV rays. Our results may therefore overstate their actual prevalence, depending on causes other than pesticide exposure [28, 34, 40, 51].

Several major limitations of the study should be addressed. For instance, we assessed a sample of relatively small size, gathered through convenience sampling and a regional basis. The latter, may represent a main issue, as Italy has been repetitively acknowledged as highly heterogeneous in terms of socioeconomically development, education level, and also agricultural practices are deeply regionalized [52]. APT in particular is associated with developed, high profitable farming, characterized by an extensive use of pesticides in small enterprises, usually managed by the owner himself. Again, it should be remarked that the study population included only subjects having a relatively high qualification, both in term of personal education and in empirical experience with pesticide handling. Moreover, most of them are either "hobby farmers", or part-time farmers: in other words, the sample we presented may not be representative of the national level [52].

Generalization of our results may be furtherly compromised by the very same retrospective conception of the survey, potentially lacking the sensibility to correctly recall cases related to pesticide toxicity both in terms of frequency and severity [7, 44, 50]. Moreover, we did not assess other professional exposures potentially related with reported symptoms, such as high environmental

temperatures, solar radiation, and also chemicals other than pesticides.

In conclusion, PAs from APT exhibited a relatively high awareness about possible adverse effects of pesticides, high levels of adoption of appropriate measures including the use of PPE and personal hygiene practices. Younger age groups and higher education were correlated with lower prevalence and frequency of symptoms, and also participants interacting with health professionals and who took part to professional courses similarly had a better awareness of the risks. The role for pesticide resellers and some information sources, including both conventional and new media, was more controversial. Professional courses, possibly involving health professionals such as occupational physicians, may therefore represent the more appropriate items to raise the awareness of farm workers towards the appropriate use of well-maintained PPE and personal hygiene practices, during and after the handling of pesticides.

## Acknowledgements

The authors express their gratitude to all participants.
Funding sources: this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Conflict of interest statement

None declared.

## Authors' contributions

MR, GG and LV equally contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript. However, as primary investigator, MR was asked to assess unclear responses of the questionnaires to determine the correct answers, whereas GG and LV performed the majority of data entry.

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■ Received on March 11, 2018. Accepted on July 30, 2018.

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ORIGINAL ARTICLE

# Isolation of *Mycobacterium tuberculosis* from livestock workers and implications for zooanthroponotic transmission in Ibadan, South-western Nigeria

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Keywords

Tuberculosis • Non-tuberculous mycobacteria • Zoonoses • Molecular characterization • Nigeria

Summary

**Introduction.** *Tuberculosis (TB) remains a public health problem in sub-Saharan Africa coupled with dearth of information about the disease among livestock workers at risk of infection. We determined the prevalence of pulmonary TB infection and factors associated with its occurrence among livestock workers in south-western Nigeria.*

**Methods.** *A cross-sectional study was conducted using active case-finding among livestock workers with sub-clinical pulmonary TB between August 2014 and March 2015. Sputum samples were cultured and subjected to a two-step multiplex-PCR technique based on genus-typing and genomic regions-of-difference. Interviewer-administered questionnaire was utilized in assessing worker's TB related knowledge and practices. Data were analysed using STATA 12.*

**Results.** *Overall, 206 livestock workers (traders = 136; butchers = 70) were screened; 5.1% (7/136) of the traders and 7.1% (5/70) of the butchers had positive mycobacterial cultures. Molecular techniques identified one Mycobacterium tuberculosis with six non-tuberculous mycobacteria (NTM) from the traders and four M. tuberculosis with one NTM from the butchers. Participants within the age range of 50 years and above were at higher risk of being infected with TB (OR = 7.7; 95% CI: 1.7-35.6) and majority had poor knowledge and practices regarding TB.*

**Conclusions.** *We confirmed M. tuberculosis as the cause of pulmonary TB among the livestock workers, with implications for zooanthroponotic transmission of the disease along the human-animal ecosystem interface in Nigeria.*

## Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is prevalent worldwide. However, occurrence of the infection is more pronounced in developing countries where surveillance and control measures are inadequate or in some cases nonexistent [1]. Research shows that one-quarter (25%) of the estimated global TB incidence occurs in the African region and majority (70%) of the top thirty countries with the highest burden of the disease are in Africa and Asia [1]. Tuberculosis has great impact on the already exacerbated health problems in sub-Sahara Africa, with estimated TB case fatality rate increasing from 5% to 20% in 2015 [1]. In Africa, the true burden of the disease is unknown largely due to poor and inefficient case reporting and documentation, limited diagnostics and laboratory infrastructure as well as non-commitment of governments [2]. Consequently, the WHO reports are mere underestimation of the true picture of the state of the disease in Africa [1, 3]. Globally, the prevalence of TB among livestock workers is not known, however studies carried out in different parts of the world have reported the isolation of *M. bovis* from livestock workers [2, 4-6]. Considering these pockets of reports of isolation of *M. bovis* from the occupationally exposed, it becomes imperative to investigate the actual prevalence of infection among this group of individuals bearing in mind the key role they play in the disease transmission chain especially in sub-Sahara Africa and other developing countries.

Nigeria, with an estimated population of 196.2 million, ranks 7$^{th}$ among countries with high TB burden, and with South Africa each accounting for 4% of global burden of the disease in 2016 [1]. In Nigeria, like majority of other African countries, despite the exposure of livestock workers (cattle rearers, traders and butchers) to cattle with bovine TB [4, 7], limited studies have investigated possible ongoing zoonotic TB infection among these workers. In addition, due to poor provisions for livestock workers within the healthcare scheme in the country, they are largely neglected and underserved thereby exposing them to a myriad of zoonotic diseases. This constitutes a serious public health concern, since these workers are often in continuous and in some instances very close contact with the general public; thus, serving as a potential source of human to human transmission of zoonotic diseases.

More importantly, these workers are generally uneducated, exhibit poor hygiene practices, indulge in alcoholism, drug addiction and have poor medical care-seeking behavior. A recent study revealed important knowledge gaps with poor practices about zoonotic TB prevention among livestock workers in Nigeria [8]. Coupled with these are the poor state of infrastructures at the cattle markets and abattoirs/slaughter slabs settings where they work [9], which are factors that could facilitate transmission of TB within the human-animal ecosystem interface in Nigeria. We therefore investigated the epidemiology of TB amongst livestock workers operating in a cattle market and an abattoir in southwestern Nigeria. Information obtained will assist in providing concrete control measures against the disease.

## Materials and methods

### Study location

The study was conducted at the Akinyele Cattle Market (ACM) and Abattoir. Akinyele is located in the northern part of Ibadan metropolis. The market is a major rallying point for cattle brought from various Northern states of Nigeria and neighbouring African countries. Due to poor infrastructure at the cattle market and abattoir, overcrowding and congestion of humans and cattle are common features [4]. Approximately, there are about 800 people within the ACM, comprising mainly of cattle traders, transporters (drivers and those who assist in offloading and loading of cattle) and members of the general public who patronize the traders. Also, present within the ACM vicinity are food vendors, veterinarians and other animal healthcare workers. These veterinarians and animal health workers constituted less than 2% of the population in the market. More importantly, the facilities are managed by largely uneducated workers and are operated under unhygienic conditions [4]. As such; transmission of zoonotic infections between humans and cattle may be easily facilitated.

### Study design, sample size and sampling

A cross-sectional study involving active case finding among livestock workers with high risk of sub-clinical pulmonary TB was carried out between August 2014 and March 2015. Using an earlier reported prevalence of 5% among butchers in Ibadan [4], the estimated sample size was 73 participants (at both the market and abattoir). Eligible participants were informed about the aim of the study and verbal/written consent was obtained from willing participants. In all, 136 and 70 participants respectively, were screened at the cattle market (traders) and abattoir (butchers). Sputum samples (one per participant) were simultaneously collected from every study participant using properly labeled sterile plastic sample containers with coded identifiers to maintain confidentiality.

### Sample processing

*Bacteriology*

Sputum samples were processed based on the Becton Dickinson digestion and decontamination procedure [10]. The final concentrate thereby obtained was inoculated onto paired Löwenstein-Jensen slopes with pyruvate and glycerol, respectively and incubated at 37ºC for 12 weeks. Isolates were harvested for genus and deletion typing by scraping the growth from a slope into 200 µl of 7H9 Middlebrook (broth) and heating at 80ºC for 1 h.

*Genus typing*

The isolates obtained were subjected to genus typing to identify members of the *M. tuberculosis* complex (MTC) as earlier described [11]. Briefly, heat-killed mycobacterial isolates from culture-positive samples were used as a DNA template. DNA amplification was done with the reaction mixture containing 2 µl DNA template, 5 µl of Q-buffer, 10X Buffer, 25 mM MgCl2, 4µl × 10 mM dNTPs, 0.5 µl of each primer (50 pmol/µl; four primer pairs were used) and 0.2 µl GoTaq Flexi DNA polymerase (Promega Madison, USA). The mixture was made up to 25 µl with ultra-pure water and heated in a Thermal Controller (MyGene Series Peltier, Model MG 96+) using the following amplification programme: 95°C for 10 min for enzyme activation, followed by 35 cycles at 95°C for 1 min for denaturation, 61°C for 0.5 min for annealing, and 72°C for 2 min for extension. After the last cycle, the samples were incubated at 72°C for 10 min. Thereafter, PCR amplification products were fractionated by electrophoresis in 3.0% agarose in 1 X TBE pH 8·3 at 6 V/cm for 4 h, and visualized by staining with Gel Red.

*Deletion typing*

The final products obtained from genus typing were further subjected to deletion typing to identify specific strains of MTC based on the presence or absence of genomic region of difference (RD) as described elsewhere [12]. Each reaction mixture consisted 1µl DNA template, 5 µl Q-buffer, 2.5 µl × 10 buffer, 2 µl 25 mM MgCl2, 4 µl × 10 mM dNTPs, 0.5 µl of each primer (50 pmol/µl), 0.125 µl GoTaq Flexi DNA polymerase (Promega Madison, USA), and was made up to 25 µl with ultra-pure water. The reaction mixture was amplified inside a Thermal Controller (MyGene Series Peltier, Model MG 96+) using; 95°C for 10 min, followed by 45 cycles at 95°C for 1 min, 61°C for 0.5 min and 72°C for 2 min. After the last cycle, the samples were incubated at 72°C for 10 min. The PCR product was finally amplified for visualization as previously described [12].

### Questionnaire survey

The knowledge and practices of the respondents about zoonotic TB were investigated using an interviewer-administered structured questionnaire. The questionnaire was pretested and modified to improve clarity. The questionnaire consists of both open and close-ended questions. Information obtained included socio-demographic characteristics

of livestock workers as well as factors associated with zoonotic TB transmission. In order to assess the knowledge and practices of respondents, scores between "zero and one" were respectively assigned to each correctly and incorrectly answered question. All questions answered completely and accurately attracted an overall score of 10 and 12 for each of the zoonotic TB prevention knowledge and practices, respectively per respondent. A knowledgeable respondent is regarded as the one who is able to score six or more out of the knowledge questions, and a respondent is said to have exhibited good practices if he/she is able to score eight or more of the practice questions. Each of these scores is equivalent to about 60% or more.

### Data analysis

Data were analysed using STATA 12 software (StataCorp, College Station, Tx). Bivariate and multivariate analyses (unconditional logistic regression) were used to test for the association between isolation of mycobacterial species and potential risk factors. Using the isolation of mycobacterial species as the outcome measure, variables with p-value of 0.20 [13] or less in the bivariate analysis and biological plausibility were included in multivariate models. Odds ratios (OR) and 95% confidence intervals (CI) were estimated. All tests were two-tailed and statistical significance was set at $p \leq 0.05$.

### Ethical approval

Ethical approval for the study was obtained from the University of Ibadan/University College Hospital Ethics Committee with approval number: NHREC/05/01/2008a. Participants were enrolled after informed consent was obtained from them. Participants who were confirmed culture positive for TB were referred to the nearest Directly Observed Treatment Short Course Centres for treatment according to the Nigerian National Guidelines for management of TB.

## Results

### Socio-demographic characteristics of study participants

More than half (66.0%) of the livestock workers screened were traders (cattle marketers) and aged between 20 and 49 years (69.9%). Most of the respondents were males (83.5%) while over half (59.7%) had formal education and were more than five years in the business (56.3%) (Tab. I).

### Bacteriology and molecular typing

A total of 12 mycobacterial species were obtained using genus typing. Seven (58.3%) were non-tuberculous mycobacteria (NTM) while five were members of the MTC family (Fig. 1). However, deletion typing showed that all the five MTC were *M. tuberculosis* (Tab. II, Fig. 2). In all, one *M. tuberculosis* and six NTM were isolated from the traders, while four *M. tuberculosis* and one NTM were recovered from the butchers (Tab. II).

### Knowledge and practice about zoonotic TB

Majority of the livestock workers had poor knowledge (62.6%) and showed poor practices (81.1%) in relation to TB (Tab. I). Only 21% of the respondents knew that man and animals could share common diseases, while 38% knew that TB could be contracted from animals. However, only 22% knew TB is curable (Tab. III). In addition, 58% of the respondents had received BCG vaccination, 18% had been tested for TB while less than half would seek modern medicine in case they have TB (Tab. IV).

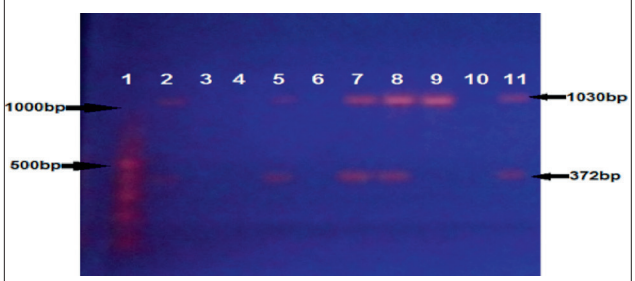
### Prevalence and associated factors of TB among study participants

The bivariate analysis revealed association between age and infection with TB. Individuals who were aged 50 years and above were at higher risk of being infected with *M. tuberculosis* (OR = 6.0; 95% CI: 1.6-22.1) (Tab. V). The multivariable logistic regression analysis identified age, educational status and knowledge about TB as positive predictors of mycobacterial species infection among livestock workers (Tab. VI). Our findings revealed that individuals older than 50 years of age were more likely to be infected when compared to those aged between 20-49

Tab. I. Characteristics of livestock workers screened for the study.

| Variables | Category | Frequency | Percentage |
|---|---|---|---|
| Population | Traders | 136 | 66.0 |
| | Butchers | 70 | 34.0 |
| Sex | Male | 172 | 83.5 |
| | Female | 34 | 16.5 |
| Age | 0-19 | 9 | 4.4 |
| | 20-49 | 144 | 69.9 |
| | ≥ 50 | 53 | 25.7 |
| Education status | No formal education | 83 | 40.3 |
| | Primary | 50 | 24.3 |
| | Secondary | 55 | 26.7 |
| | Tertiary | 18 | 8.7 |
| Duration in business | 0-5 | 90 | 43.7 |
| | 6-10 | 66 | 32.0 |
| | ≥ 11 | 50 | 24.3 |
| Knowledge | Poor | 129 | 62.6 |
| | Good | 77 | 37.4 |
| Practices | Poor | 167 | 81.1 |
| | Good | 39 | 18.9 |

Fig. 1. Genus typing banding pattern of isolates Lane 1 = DNA ladder; Lanes 2, 5, 7 & 8 = MTC; Lane 9 = NTM; Lane 10 = negative control; Lane 11 = positive control (H37Rv).

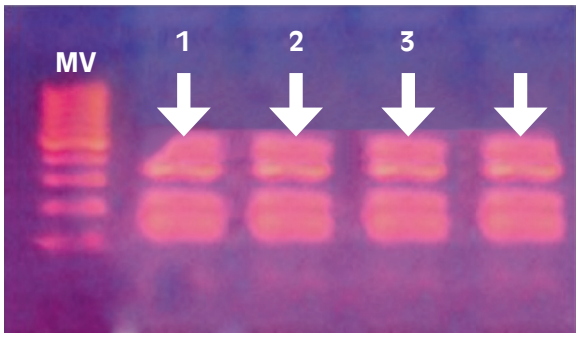


**Tab. II.** Number of mycobacterial isolates cultured and confirmed from livestock workers.

| Diagnostic tools | Traders | Butchers | Total |
|---|---|---|---|
| Culture | 7 | 5 | 12 |
| Genus typing | 1 MTC & 6 NTM | 4 MTC & 1 NTM | 5 MTC & 7 NTM |
| Deletion typing | 1 Mtb | 4 Mtb | 5 Mtb |

MTC: Mycobacterium tuberculosis complex; NTM: non-tuberculous mycobacteria; Mtb: Mycobacterium tuberculosis.

**Fig. 2.** Deletion typing banding pattern for MTC isolates. Lane 1, 2, 3 = Mycobacterium tuberculosis; Lanes 4= positive control (H37Rv).




years (OR = 7.7; 95% CI: 1.7-35.6). In addition, those who had secondary school education were less likely to be infected when compared to those without formal education (OR = 0.1; 95% CI: 0.0-0.8). Likewise, individuals with good knowledge of TB were less likely to be infected when compared to those with poor knowledge of the disease (OR = 0.1; 95% CI: 0.0-0.1) (Tab. VI).

## Discussion

This study sets out to confirm the possible infection of livestock workers with members of the MTC particularly *M. bovis* given their high risk of exposure to BTB. Coincidentally, instead of *M. bovis*, five cases of *M. tuberculosis* infection were confirmed among livestock traders and butchers with sub-clinical pulmonary TB infections. Since most of these workers work and live under very poor sanitary and environmental health conditions and were unaware of TB, there was increased risk of contracting the disease from infected fellow workers and/or cattle. While we had initially hypothesized *M. bovis* infection among these workers given an earlier report of zoonotic infection in the same setting [4], this was not the case in this study as we only confirmed cases of *M. tuberculosis* infections. This notwithstanding, our findings are of public health importance, since those infected would have remained unidentified and untreated, with the likelihood of spreading the infection to fellow workers and household members. More notably, this becomes important given the poor health care-seeking behaviour in Nigeria and other low-middle income countries [4, 14-16] which may aggravate the burden of TB in these countries.

Poor knowledge of TB has earlier been identified as an important risk factor among hospital patients and the occupationally exposed group [4, 17, 18]. In our study, we observed that majority of the participants had poor knowledge of TB and exhibited practices that could facilitate spread of disease within the abattoir and cattle market settings. Importantly, poor knowledge of TB was significantly associated with *M. tuberculosis* infection among the study participants while individuals who had good knowledge of the disease were less likely to be infected. As observed, a little above half of the respondents had received BCG vaccination despite the fact that this vaccination is recommended for every individual at birth in Nigeria. It is therefore of concern that such a relatively high proportion of these occupationally exposed individuals had not received this vaccination. Worse still, less than half indicated they would seek modern medicine in case they had TB. This suggests that the level of awareness of TB treatment is still low among the occupationally exposed individuals who are at higher risk of the infection.
One major important public health implication of our findings is the possibility of zooanthroponotic infection of cattle and other animals with *M. tuberculosis* in the study setting. Though our current study did not involve simul-

**Tab. III.** Knowledge of zoonotic TB among livestock workers in Ibadan, Nigeria.

| S/N | Questions | Response options | % |
|---|---|---|---|
| B1 | Do you know that man and animals can share common diseases? | Yes<br>No | 21<br>89 |
| B2 | What type of disease can be contracted? | TB<br>Cancer<br>Ebola<br>AIDS | 28<br>14<br>36<br>22 |
| B3 | If no, why do you think that man cannot contract disease from animals? | Don't know<br>Not possible | 44<br>56 |
| B4 | Do you know that someone can contract TB from animals? | Yes<br>No | 38<br>62 |
| B5 | How do you think man can be infected with TB? | Direct contact<br>Consumption of meat and milk products<br>Aerosol | 46<br>37<br>17 |
| B6 | Do you know the clinical signs in processed animals? | Yes<br>No | 18<br>82 |
| B7 | What are signs/ symptoms of TB you will observe in a person with TB? | Cough<br>Wasting<br>Sweat in the night<br>Body weakness | 36<br>24<br>17<br>23 |
| B8 | Is TB curable? | Yes<br>No<br>Don't know | 22<br>48<br>30 |
| B9 | What type of treatment is best for TB? | Modern medicine<br>Traditional medicine<br>Faith healing | 46<br>34<br>20 |
| B10 | Boiling milk before consumption destroy the bacteria causing TB | Yes<br>No<br>Don't Know | 24<br>27<br>48 |

**Tab. IV.** Practices related to zoonotic TB among livestock workers in Ibadan, Nigeria.

| S/N | Questions | Response options | % |
|---|---|---|---|
| C1 | Have you received BCG vaccination | Yes<br>No | 58<br>42 |
| C2 | What do you do to protect yourself from animal infections? | Immunization<br>Use of preventive medicine<br>Good hygiene practices<br>Use of herbs | 15<br>50<br>25<br>10 |
| C3 | Have you ever been tested for TB | Yes<br>No | 18<br>82 |
| C4 | What would you do if you see your slaughtered animal or carcass having TB lesions? | I will sell it<br>I will kill it and bury it<br>I will eat it personally at home | 52<br>26<br>22 |
| C5 | If you have TB where would you go for treatment? | Modern medicine<br>Traditional medicine<br>Faith healing | 42<br>38<br>20 |
| C6 | What type of hygiene practice do you observe to protect yourself from contracting TB from animals? | Putting on protective material while working<br>Limited contact with animals or carcass<br>Washing of hands after touching live or processed animals<br>None of the above | 8<br>12<br>20<br>60 |
| C7 | Do you boil raw cow milk before drinking? | Yes<br>No | 12<br>88 |
| C8 | Do you cohabit with your animals? | Yes<br>No | 91<br>9 |
| C9 | Do you pack animal dungs with bare hands | Yes<br>No | 89<br>11 |
| C10 | Do you process or handle animal carcasses with unprotected wounds? | Yes<br>No | 57<br>43 |
| C11 | What disease condition have you experienced recently? | Cut/sores<br>Cough lasting more than three weeks<br>Wasting<br>None of the above | 45<br>21<br>14<br>20 |
| C12 | Do you need to go for medical check -up periodically? | Yes<br>No | 84<br>16 |

taneous screening of cattle for *M. tuberculosis* and other MTC, we cannot preclude the possibility of animal infections with *M. tuberculosis* given the prevalent unhygienic and close human-animal interactions within the setting. More so, earlier reports in Nigeria, Spain, some countries in Africa and Asia [4, 7, 18-20], indicated several cases of animal infections with *M. tuberculosis*. These might not be unconnected with similar deplorable environmental conditions that support zooanthroponotic infections with *M. tuberculosis* given high probability of the risk of pulmonary route of TB transmission/infection among livestock workers and cattle in these settings. More importantly, since the prevalence of TB is high among humans in Nigeria, there is high risk of animal infection and the risk of spill back to humans; thus, posing public health risk via the food chain.

**Tab. V.** Prevalence of TB among livestock workers screened for the study.

| Variables | TB infection | | OR | 95%CI | P-value |
|---|---|---|---|---|---|
| | Positive n (%) | Negative n (%) | | | |
| Population | | | | | |
| Traders | 7 (58.3) | 123 (65.4) | 1.0 | - | - |
| Butchers | 5 (41.7) | 65 (3346) | 1.2 | 0.3-3.8 | 0.86 |
| Sex | | | | | |
| Male | 9 (75.0) | 163 (84.0) | 1.0 | - | - |
| Female | 3 (25.0) | 31 (16.0) | 1.8 | 0.4-6.8 | 0.67 |
| Age | | | | | |
| 0-19 | 0 (0.0) | 9 (4.6) | - | - | - |
| 20-49 | 8 (66.7) | 170 (87.6.) | 1.0 | - | - |
| ≥ 50 | 4 (33.3) | 15 (7.7) | 6.0 | 1.6-22.1 | 0.01 |
| Education status | | | | | |
| No formal education | 8 (66.7) | 78 (40.2) | 1.0 | - | - |
| Primary | 2 (16.7) | 48 (24.7) | 0.4 | 0.1-2.0 | 0.42 |
| Secondary | 1 (8.3) | 51 (26.3) | 0.2 | 0.0-1.6 | 0.17 |
| Tertiary | 1 (8.3) | 17 (8.8) | 0.6 | 0.1-4.9 | 0.95 |
| Duration in business | | | | | |
| 0-5 | 3 (25.0) | 87 (44.9) | 1.0 | - | - |
| 6-10 | 4 (33.3) | 62 (32.0) | 1.9 | 0.4-8.7 | 0.67 |
| ≥ 11 | 5 (41.7) | 45 (23.2) | 3.2 | 0.7-14.1 | 0.21 |
| Knowledge | | | | | |
| Poor | 10 (83.3) | 121 (62.4) | 1.0 | - | - |
| Good | 2 (16.7) | 73 (37.6) | 0.3 | 0.1-1.5 | 0.24 |
| Practices | | | | | |
| Poor | 10 (83.3) | 159 (82.0) | 1.0 | - | - |
| Good | 2 (16.7) | 35 (18.0) | 1.9 | 0.5-7.4 | 0.62 |

**Tab. VI.** Multivariable logistic regression analysis of variables significant at 20% level with the main outcome measure (TB infection) in bivariable analysis.

| Variables | Category | OR | 95%CI | P-value |
|---|---|---|---|---|
| Age | 0-19 | - | - | - |
| | 20-49 | 1 | - | - |
| | ≥ 50 | 7.7 | 1.7-35.6 | 0.01 |
| Education | No formal education | 1 | - | - |
| Status | Primary | 0.3 | 0.1-1.7 | 0.17 |
| | Secondary | 0.1 | 0.0-0.8 | 0.04 |
| | Tertiary | 1.4 | 0.1-15.5 | 0.76 |
| Knowledge | Poor | 1 | - | - |
| | Good | 0.1 | 0.0 – 0.1 | 0.02 |

Also, findings from this study revealed that the proportion of mycobacterial species recovered from the abattoir workers was higher than that from the livestock traders. Notably, more *M. tuberculosis* were isolated from the butchers (four) compared to the traders (one). The higher cases among abattoir workers could be attributed to the more congested work place setting obtainable within the enclosed slaughter house facility as compared to the open environment used by the livestock traders, even though overcrowded. Incidentally, this factor has been implicated in the human-to-human transmission of TB in earlier studies [4, 21].

Further, our findings showed that age was a risk factor for mycobacterial infection among study participants with individuals who were 50 years and older having

higher likelihood of being infected. Association between age and mycobacterial infection has earlier been reported [22]. Factors associated with old age such as duration of time spent in business and immunocompromised state can predispose this group of individuals to higher risk of infection [4]. In high income countries, it has long been acknowledged that older people are vulnerable to developing TB [23].This fact is seldom recognized in developing countries largely due to factors such as non-specific clinical presentations, knowledge gap about TB, non-consideration of the disease at this extreme age, and late diagnosis [23, 24]. The significance of old age in the epidemiology of TB in humans was reiterated by the 2010 Global Burden of Disease estimates which reported that 57% of all TB deaths worldwide occur among individuals older than 50, with a little more than half of this occurring among 65 and above [23, 25]. In addition, older people (50 years and above) have been identified to account for a large percentage (34%) of Disability-Adjusted Life Years (DALYs) attributable to TB globally [23, 25].

Besides, our results identified educational status as a significant risk factor for TB infection. The individuals with secondary school education were less likely to be infected with TB than those who did not have formal education. Our finding is consistent with previous studies [26, 27] that associated educational status with TB infection. Furthermore, previous reports [28, 29] posited that there exists a direct link between knowledge of TB, which is usually driven by level of education, and risk of being infected. Again, adherence to basic hygiene and protective measures against TB was reported to be common among educationally exposed professionals compared to their non-educated counterparts [17, 27]; thus, making them less exposed to risk of TB infection.

Importantly, we isolated NTM from livestock workers, underscoring the relevance of this group of pathogens in the epidemiology of TB in humans. Several reports have continually linked NTM to pathogenesis of mycobacterial infections in humans causing several clinical manifestations [28-31]. Our finding is of public health concern, especially in Nigeria where laboratory diagnosis of TB is mostly based on smear microscopy which cannot differentiate between MTC and NTM. Consequently, there is a greater tendency of misdiagnosing NTM as MDR-TB since NTM will not ordinarily resolve using the conventional treatment regimen of MTC [29, 32]. Unfortunately, this leads to smear positive patients being placed on MDR-TB second line drugs which are very toxic [33-35].

Despite the findings of this study, there were some limitations. First, we did not carry out molecular typing techniques like spoligotyping and variable number of tandem repeats (VNTR) to further characterize the isolated *M. tuberculosis* and compare them with strains on existing database. Again, we did not carry out drug susceptibility testing on the isolates to confirm their susceptibility patterns. In all, these would have provided some insight into whether they are MDR-TB strains given this emerging public health problem in Nigeria [1]. Also, we did not simultaneously screen cattle from the livestock market and abattoir where the participants were screened. Evidence of direct epi-link on possible ongoing zooanthroponotic transmission of *M. tuberculosis* between the livestock workers and cattle would have been revealed. Though these were beyond the immediate scope of the present study, future epidemiological studies will take care of these gaps.

## Conclusions

We report the isolation of *M. tuberculosis* and NTM among livestock workers suspected to have sub-clinical pulmonary TB infections in Ibadan, Nigeria. We also show that *M. tuberculosis* and not *M. bovis* was the incriminating agent responsible for TB among livestock workers in the current study. Furthermore, we identified older age, low educational status and poor knowledge of TB as significant risk factors associated with the infection among livestock workers. Given poor knowledge and practices of these workers towards TB, health threats along the human-animal ecosystem interface may promote zooanthroponotic transmission of *M. tuberculosis* to cattle. On this premise therefore, we advocate a multidisciplinary approach using the One-Health umbrella (i.e. a multidisciplinary platform involving veterinarians and human medics) to provide more insights into zoonotic TB transmission and mitigate its public health threats. There is need for the Ministry of Health in Nigeria to step up awareness campaigns regarding tuberculosis and availability of treatment particularly among the occupationally exposed individuals. Finally, we reiterate the importance of molecular typing methods in the proper identification of MTC and other mycobacterial infections among infected humans and cattle along the human-animal ecosystem interface.

## Acknowledgements

This project was partly supported by D. John and Catherine T MacArthur Foundation, USA under the Higher Education Initiative in Africa (Grant No. 97944-0-800/406/99) for the establishment of a center of Excellence that supports this Consortium of Universities.

## Conflict of interest statement

None declared.

## Authors' contributions

SC conceived the idea of the study and approved the final draft of the manuscript.VA wrote the first draft of the manuscript and carried out the data analysis. AA, NO, MA, JO, HA, EC and VA coordinated screening and sample collection among the livestock workers and writing of the manuscript.

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■ Received on August 2, 2018. Accepted on August 15, 2018.

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ORIGINAL ARTICLE

# Availability and utilization of sanitation facilities in Enderta district, Tigray, Ethiopia

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Keywords

Availability • Utilization • Sanitation • Water • Ethiopia

Summary

**Introduction**. *Despite concerted efforts by governmental and non-governmental organizations, water and adequate sanitation still remain a challenge worldwide. Therefore, this study assessed the availability and utilization of sanitation facilities in Enderta district of Tigray, Ethiopia.*
**Methods**. *A cross-sectional study was conducted in May 2016. An interview and observation were conducted in a total of 450 households. An interviewer administered questionnaire and observation checklist were used to collect the data. Multivariable logistic regression was done to identify the predictors of availability and utilization of latrine, hand washing, and drinking water.*
**Results**. *Out of 450 households, only 68.4% had latrine of which only 21.4% had hand washing facility near the toilet. Likewise, only 9.3% washed their hand after toilet. However, all households had access to improved water source. In this study, proper utilization of latrine, hand washing, and water facilities was observed in 53%, 42.4% and 36.2% of the study households, respectively. The family size and getting sanitation information from health care providers, health extension workers, and health development army had a positive effect on availability and utilization.*
**Conclusions**. *Though Community-Led total sanitation and hygiene approach has been implemented in the study area, the availability and proper utilization of latrine, and hand washing are still low. Only few households used drinking water properly. The education or information on hygienic practices found to affect the availability and utilization of the sanitation facilities positively. Therefore, strengthening the health extension workers and health development army to provide sustainable education and health information is needed.*

## Introduction

Adequate drinking water, proper sanitation, and hygiene are essential requirements to ensure human health and better economic development [1]. Currently, the importance of water and adequate sanitation is recognized at both local and global levels. In spite of concerted efforts by governmental and non-governmental organizations, water and adequate sanitation are still remained a challenge. Globally, 2.4 billion people still lack improved sanitation facilities, and more than half billion people lack access to improved drinking water sources [2]. And, sub-Saharan Africa is the region where most of these people live. In Ethiopia, though there is a significant improvement in access to water and sanitation facilities, the sanitation coverage is still minimal [3]. An estimated 47.5% and 72% of the population lack access to improved drinking water source and sanitation facilities, respectively [2, 4]. Moreover, open defecation is still practiced by more than a quarter (29%) of the population [5, 6]. In Tigray region, the area in which the current study conducted, the open field defecation rate is 35.6% which is slightly higher than the national level [5]. Further, in 2011 the latrine coverage was 87%, though the utilization rate was very minimal (34%) in the region [7]. However, a higher latrine utilization rate (57.3%) was reported in one of the districts of the region since 2013 [8].

Diarrheal diseases represent the most significant health impact of unimproved sanitation, and extremely high impact upon children [9]. Globally, inadequate sanitation, poor hygiene, and unsafe drinking water contribute to 88% of diarrheal disease [9-11]. Moreover, diarrheal diseases are the second leading cause of death in children under the age of five, estimated that 1.5 million child deaths annually. Severe diarrhea may be life threatening due to fluid loss, particularly in infants, young children, the malnourished and people with impaired immunity such as those living with Human Immunodeficiency Virus (HIV). Likewise, the impact of poor sanitation, hygiene, and unsafe drinking water is also recognized to have a positive effect on the burden of neglected tropical diseases such as trachoma, dracunculiasis and visceral leishmaniasis [12].

In Ethiopia, 60% of overall diseases are related to poor sanitation and lack of hygiene. Diarrhea is the leading cause of under-five mortality causing 23% of all under-five deaths in the country [13]. By improving water, sanitation, and hygiene about 64,540 children could be saved every year in the country. About 40% of under-five children in Ethiopia are stunted, which can be strongly linked to the childhood incidence of diarrhea [13, 14].

The Community-led Total Sanitation and Hygiene (CLTSH) approach has been proven as one of the cost effective approaches to improve the water, sanitation, and hygiene worldwide especially for those with low-income. Thus, with the goal of ending "open defecation" through self-built toilets, and by encouraging appropriate hand washing and water handling practices; the government of Ethiopia has been implementing CLTSH by integrating it with the health extension program in all the nine regions since 2011 [15]. However, political commitment at all levels had focused on sanitation coverage, with less attention given to improving safe water, sanitation, and hygiene practices such as hand-washing, safe water storage and handling, and latrine maintenance and usage [16]. As a result, with all the efforts, the prevalence of sanitation related diseases is still high in the country [17].

There are few studies conducted in Ethiopia [8, 18-21]. However, these studies were focused on larine utilization and they were conducted at the time when the CLTSH was not implemented or too child in the country. They were only focused on utilization of latrine but all issues regarding sanitation and drinking water were not addressed fully. However, the current study was conducted after five years of CLTSH implementation thus the findings could show us the improvement brought by the program. Therefore, the aim of this study is to assess the availability and utilization of sanitary facilities in CLTSH implemented district of Tigray region, Ethiopia. The finding of the study, therefore, will help to design evidence based strategy to enhance availability and utilization of sanitary facilities on the study area. Hence, the CLTSH implementation will be further strengthened.

## Methods

### Study area and design

A community-based cross-sectional study was conducted in May 2016 in Enderta district. The district is located at the 795 kms north of Addis Ababa, the capital of Ethiopia, and about 12 kms southeast of the Tigray Regional city, Mekelle. The district comprises 17 kebeles (the smallest administrative unit in Ethiopia) and 60 villages. In 2015, the district had a total of 112,154 and 25,489 population and households, respectively. There are 6 health centers, 11 health posts, and 664 health development armies with the total health staffs of 170, of which 35 are Health Extension Workers (HEWs).

### Study population

Randomly selected households of the rural community who owned private latrine were the study population for this study.

### Sample size, sampling technique and procedure

The sample size was determined using single population formula with 2015 estimates of access to sanitation 28% [2], with a margin of error of 0.05% at the 95% confidence level. Then, multiplying by a design effect of 1.5, the total sample size was calculated to be 450. A multistage sampling technique was employed to select the sample households. Primarily, three kebeles were selected randomly. From each kebele, one village was selected randomly. Then, the sample of 450 households was selected based on proportional to population size (PPS) allocation to each of the selected villages. The households were selected by systematic sampling method from the already prepared sampling frame. Then, in the selected households, the household heads were recruited and observations were done.

### Data collection tools and procedure

Data collection tools such as interviewer administered questionnaire and observation checklist were used to collect data from the households. The data collection tools include socio-demographic and other characteristics that would measure the availability and utilization of sanitation facilities and associated factors after reviewing relevant literatures.

### Operational definitions

Presence of sanitation and hygiene facilities such as a latrine, hand washing, and water source was considered as availability. Households were considered as properly utilizing latrine if the latrine is not shared, pit with slab/cover, the compound is free of observable faeces and has functional latrine during the study, having no observable faeces around the squat hole [22]. Households were considered as properly practice hand washing if there was hand washing material with water and soap/or ash during the data collection period. In this study, a household was considered as properly handling water, if the container is clean, has cover and placed away from any source contamination such as animals during the data collection period [22].

### Data quality control

Prior to data collection training was given to data collectors and supervisors on the content, objective and methods of data collection and interviewing techniques. The pretest was done in 5% of the sample from villages with the population having similar socio-demographic characteristics that were not included in the study and minor correction was done accordingly. The questionnaire was also translated into local language (Tigrigna) and back-translated to English to ensure the consistency of the thought of the questions. During data collection time, a clear introduction that explained the purpose and objectives of the study were provided to respondents. A close supervision, honest communication and on spot decisions were made during data collection.

Validity and reliability of the questionnaire was determined as follows. Face and content validity of the tool was determined based on viewpoints of the experts on the area. Test-retest reliability of the tool was examined by pre-testing the tool on 5% of the sample size in similar context with 10-12 days interval. Hence, the questionnaire was reviewed and analysed for repeatabil-

ity and internal consistency aspects. Cronbach's alpha coefficient was also used to assess internal consistency and said to be internally consistent if score of 0.8 and above. Repeatability was estimated using the intra-class correlation coefficient (ICC). Based on ICC reliability ranges of less than 0.4 (poor), 0.4-0.7 (fair to good), 0.6-0.8 (good) and 0.8-1 (excellent), the reliability of the questionnaire was considered excellent.

### Data management and analysis

Quantitative data were checked for completeness, edited, coded, entered and analyzed using STATA version 13 (Stata Corp. Texas, USA). The descriptive summary was done using frequencies and proportions. Multivariable logistic regression was done to identify the independent predictors of availability and utilization of sanitation facilities and drinking water. The strength of association was measured by odds ratios at their 95% Confidence levels and the statistical significance was set at the p-value of 0.05.

### Ethical consideration

Ethical approval was obtained from the Institutional Review Board of College of Health Sciences, Mekelle University (EBC-06128/2016). Verbal consent was obtained from each study participants after the information about the study like the objective was explained. The participants were informed that it was their right to refuse or withdraw from the study at any point during the course of study. Names and other personal information which could violate the confidentiality of respondents were not recorded and information was kept confidential.

## Results

### Socio-demographic characteristics of household heads

A total of 450 households were included in the study with 100% response rate. Of the total respondents, 323 (71.8 %) were female headed and 334 (74%) were married. More than half (56%) of households had more than 4 family sizes. About 82% (370) household heads were illiterate (Tab. I).

### Availability and utilization of sanitation facilities

Out of the total observed households (N = 450), 68.4% respondents had the latrine, of which 45.8% were with a cover of the hole. About six in ten (86.7%) households had no faeces on the wall, floor and/or door, and 85.1% households had no faeces on their compound. Sixty-six (21.4%) had hand washing facility near to the toilet. However, fewer than half (42%) of hand washing facilities were with ash/soap. Although all households were using public piped hand pump water and 98.4% of them had a water container with cover, in 61.8% of them the water container was not hygienic. Three hundred ninety-three (87.8%) of the observed Households keep their drinking water away from animals (Tab. II). The overall proper utilization of latrine, hand washing and water were 53.3%, 42.4%, and 36.2%, respectively.

### Hand washing practice

Regarding hand washing practice during critical times, only 9.3% of household wash their hands after toilet. Similarly, lower proportions of households, 27.8%, and 34.2%, washed their hands before feeding their child and after cleaning their child, respectively. Whereas, most of the households, 83.3%, and 90.4%, washed their hands before preparing and eating their food, respectively (Fig. 1).

### Factors associated with availability of sanitation facilities

Family size and getting information or education about sanitation by either health professionals or health extension workers and health development army were positively associated with the availability of latrine. Households who had more than or equals to four family size had 72% (AOR = 1.72; 95% CI (1.13, 2.61)) higher odds of having latrine than those households that had less than four family size. According to trend chi-square test, there was increasing in the trend of having latrine as the number of sources of information about sanitation is increased (P-value < 0.001) (Tab. III). Households that have more than four families had 80% higher [AOR = 1.8; 95% CI (1.00, 3.32)] odds of having hand washing facility than their less than four counterparts.

### Factors associated with utilization of sanitation facilities

Having hand washing facility, properly use water and informed about sanitation by health development Army (HAD) were positively associated with latrine utiliza-

**Tab. I.** Background characteristics of household heads in Enderta Woreda, Ethiopia, 2016 (n = 450).

| Characteristics | Frequency | % |
|---|---|---|
| Family size | | |
| 1-4 | 197 | 43.8 |
| > 4 | 253 | 56.2 |
| Sex (HH head) | | |
| Male | 127 | 28.2 |
| Female | 323 | 71.8 |
| Marital status | | |
| Single | 60 | 13.3 |
| Married | 334 | 74.2 |
| Divorced | 26 | 5.8 |
| Widowed | 30 | 6.7 |
| Occupational status | | |
| Farmer | 422 | 93.8 |
| Daily laborer | 12 | 2.7 |
| Merchant | 10 | 2.2 |
| Government employee | 6 | 1.3 |
| Educational status | | |
| Illiterate | 370 | 82.2 |
| Primary | 29 | 6.4 |
| Secondary & above | 51 | 11.3 |

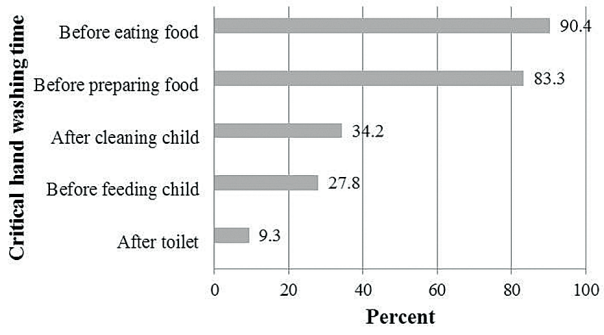
HH: household.

Tab. II. Availability and utilization of sanitation facilities Enderta Woreda, Ethiopia, 2016.

| | Sanitation facilities | Variables | Frequency | % |
|---|---|---|---|---|
| Availability | Latrine | Latrine (n = 450) | | |
| | | No | 142 | 31.6 |
| | | Yes | 308 | 68.4 |
| | Hand washing | Hand washing facility near the latrine (n = 308) | | |
| | | No | 242 | 78.6 |
| | | Yes | 66 | 21.4 |
| | Water | Presence of improved drinking water source[a] | | |
| | | Yes | 450 | 100 |
| Utilization | Latrine (n = 308) | Latrine hole has cover/slab | | |
| | | No | 103 | 33.4 |
| | | Yes | 205 | 66.6 |
| | | Absence of faeces on the wall, floor, and door | | |
| | | No | 41 | 13.3 |
| | | Yes | 267 | 86.7 |
| | | Absence of faeces on the compound of HHs | | |
| | | No | 46 | 14.9 |
| | | Yes | 262 | 85.1 |
| | | Latrine shared | | |
| | | Yes | 41 | 13.1 |
| | | No | 267 | 86.9 |
| | Hand washing (n = 66) | Presence of water for hand washing | | |
| | | No | 23 | 34.8 |
| | | Yes | 43 | 65.2 |
| | | Presence of soap/ash on the hand washing | | |
| | | No | 38 | 57.6 |
| | | Yes | 28 | 42.4 |
| | Water (n = 450) | Is water container covered | | |
| | | No | 8 | 1.6 |
| | | Yes | 442 | 98.4 |
| | | Is water container hygienic/dipper not put on floor | | |
| | | No | 278 | 61.8 |
| | | Yes | 172 | 38.2 |
| | | Is drinking water away from domestic animals | | |
| | | No | 55 | 12.2 |
| | | Yes | 395 | 87.8 |

[a]: all households had public piped hand pump water.

Fig. 1. Hand washing practice in the critical times in households of Enderta district, Tigray, Ethiopia, 2016 (n = 450).




tion. Households having hand washing facility had 2.53 times [AOR = 2.53; 95% CI (1.38, 4.59)] higher odds to utilize latrine than households that did not have hand washing facility. Likewise, those who properly use water had 97% higher [AOR = 1.97; 95% CI (1.22, 3.20)] odds to use latrine than households who did not use water properly. Households who got information/education about sanitation had 2.31 times [AOR = 2.31; 95% CI (1.39, 3.86)] higher odds to use latrine properly than their counterparts (Tab. IV).

Getting information about sanitation from HEWs was positively associated with utilization of hand washing facility. Moreover, getting information about sanitation from HEWs, using the latrine and visited by the community sanitation committee were positively associated with proper utilization of water. Households who used latrine properly had 2.07 times [AOR = 2.07; 95% CI (1.27, 3.38)] higher odds of using water properly than those who did not use latrine (Tab. IV).

## Discussion

The current study found that 68.4% of the household had latrine though only 21.4% of these households had hand washing facility near the latrine. Our finding is comparable with the 2015 national sanitation review report that reported 68% and 61% of latrine coverage nationally and in Tigray region, respectively [5]. The present study also showed that all the households in the study

**Tab. III.** Factors associated with availability of sanitation facilities in Enderta district, North Ethiopia.

| Variables | Availability of latrine | | $X^2$-test | AOR (95% CI) |
|---|---|---|---|---|
| | No: n (%) | Yes: n (%) | | |
| **Family size** | | | | |
| 1-4 | 75 (38.1) | 122 (61.9) | 1 | 1 |
| > 4 | 67 (26.5) | 186 (73.5) | 6.9** | 1.72(1.13, 2.61)* |
| **Informed by HPs** | | | | |
| No | 18 (50.0) | 18 (50.0) | 1 | 1 |
| Yes | 124 (29.9) | 290 (70.1) | 6.2* | 2.96 (1.44, 6.09)** |
| **Informed by HEWs** | | | | |
| No | 100 (41.2) | 143(58.8) | 1 | 1 |
| Yes | 42 (20.3) | 165(79.7) | 22.5*** | 2.21 (1.40, 3.47)** |
| **Informed by HDAs** | | | | |
| No | 121 (37.0) | 206 (63.0) | 1 | 1 |
| Yes | 21 (17.1) | 102 (82.9) | 16.4*** | 2.32 (1.31, 4.09)** |
| **Source of information about latrine** | | | | |
| Not heard from any of the (HP, HEW, HDA) | 5 (55.6) | 4 (44.4) | 5.9[a]*** | - |
| Heard from one of (HP, HEW, HDA) | 99 (43.4) | 129 (56.6) | | |
| Heard from two | 26 (21.1) | 97 (78.9) | | |
| Heard from three of them | 12 (13.3) | 78 (86.7) | | |
| | Availability of hand washing facility | | $X^2$-test | AOR (95% CI) |
| | No: n (%) | Yes: n (%) | | |
| **Family size** | | | | |
| 1-4 | 103 (84.4) | 19 (15.6) | 4.1* | 1.8 (1.00, 3.32)* |
| > 4 | 139 (74.7) | 47 (25.3) | | |
| **Informed by HDAs** | | | | |
| No | 152 (73.8) | 54 (26.2) | 8.5** | 0.37(0.19, 0.74)** |
| Yes | 90 (88.2) | 12 (11.8) | | |

HPs: health professionals; HDAs: health development armies; HEWs: health extension workers; AOR: adjusted odds ratio; [a]: trend Chi-square test; significant at p-value: *< 0.05, **< 0.01, ***< 0.001.

**Tab. IV.** Factors associated with utilization of sanitation facilities in Enderta district, North Ethiopia.

| Variables | Utilization of latrine | | $X^2$-test | AOR (95% CI) |
|---|---|---|---|---|
| | No: n (%) | Yes: n (%) | | |
| **Hand washing facility** | | | | |
| Not available | 123 (50.8) | 119 (49.2) | | 1 |
| Available | 21 (31.8) | 45 (68.2) | 7.52** | 2.53 (1.38, 4.59)** |
| **Proper water utilization** | | | | |
| No | 98 (53.3) | 86 (47.7) | | 1 |
| Yes | 46 (37.1) | 78 (62.9) | 7.77** | 1.97 (1.22, 3.20)** |
| **Informed by HDAs** | | | | |
| No | 107 (51.9) | 99 (48.1) | | 1 |
| Yes | 37 (36.3) | 65 (63.7) | 6.72** | 2.31 (1.39, 3.86)** |
| | Utilization of hand washing facility | | $X^2$-test | AOR (95%CI) |
| | No: n (%) | Yes: n (%) | | |
| **Informed by HEWs** | | | | |
| No | 28 (70.0) | 12 (30.0) | | 1 |
| Yes | 10 (38.5) | 16 (61.5) | 6.41* | 3.73 (1.32, 10.56)* |
| | Proper utilization/ handling of water | | $X^2$-test | AOR (95% CI) |
| | No: n (%) | Yes: n (%) | | |
| **Informed by HDAs** | | | | |
| No | 207 (63.3) | 327 (36.7) | | 1 |
| Yes | 80 (65.0) | 43 (35.0) | 0.11 | 0.57 (0.33, 0.99)* |
| **Informed by HEWs** | | | | |
| No | 178 (73.3) | 65 (26.7) | | 1 |
| Yes | 109 (52.7) | 98 (47.3) | 20.5*** | 2.05 (1.23, 3.42)** |
| **Latrine utilization** | | | | |
| No | 98 (68.1) | 46 (31.9) | | 1 |
| Yes | 86 (52.4) | 78 (47.6) | 7.77** | 2.07 (1.27, 3.38)** |
| **Visited by sanitation committee** | | | | |
| No | 94 (75.8) | 30 (24.2) | 1 | 1 |
| Yes | 193 (59.2) | 133 (40.8) | 10.72** | 2.23 (1.27, 3.89)** |

HDAs: health development armies; HEWs: health extension workers; AOR: adjusted odds ratio; significant at p-value: *< 0.05, ** < 0.01, ***< 0.001.

area had access to improved water source (public stand hand pump) [2]. However, the proper utilization was low in which 53%, 42.4% and 36.2% of households utilize latrine, hand washing, and drinking water properly, respectively. The current finding is consistent with the previous finding reported in 2013 as 57.3%, and 61.2% of latrine utilization rate [8, 20]. Besides, the present finding clearly indicates the availability of the latrine could not be a guarantee for proper utilization.

Though more than two-third of households had a latrine, only a few (9.3%) households wash hands after visiting toilet that is much lower than the nationally reported 45% [5]. Further, low proportions of households washed their hands before feeding the child (27.8%) and after cleaning their child (34.2%). However, the present study revealed higher proportions of households wash their hands before preparing (83.3%) and eating (90.4%) food which is consistent with national survey report of 2016 in which 96% of households wash their hands before eating food.

The current study also found that family size and getting information or education about water, sanitation, and hygiene from either health professionals or health extension workers or HDA were positively affected the availability of latrine in the households. That is, the households who had more than four family members had higher odds of having latrine than their counterparts. This is consistent with the finding from Ethiopia in which households who had been visited by health professionals are more likely to construct latrine and good sanitation practice [18]. Also, it was found that the size of the family was positively related with the availability of hand washing facility. The households that have more than four family members had 80% higher odds of having hand washing facility near the toilet than those who have less than four family members. Regarding utilization, households who had hand washing facility near their toilet were 2.5 times likely to use latrine properly. Likewise, those who use water properly had 97% higher odds to utilize latrine properly than their counterparts. Providing health information about sanita-

tion through HDA, HEWs, and health professionals had a positive effect on proper utilization of latrine, hand washing, and water. In line with the present finding, it is evidenced that having materials to build the latrine and to improve latrine utilization have a positive effect on the utilization of latrine [8]. Further, it has been evidenced that households educated or get informed in hygienic practices are more likely to practice latrine, water, and hygiene properly [23].

Being a model in healthy practice is evidenced to enhance the community to practice healthy behaviors [24, 25]. Likewise, in the present study, the households that used latrine properly had 2 times higher odds of utilizing water properly than those who had improper latrine use. Visit by community sanitation committee was found to have a positive effect on proper utilization of water. In line with this, a study conducted in 2015, in Ethiopia shows the households need sustainable information and education in order to utilize latrine, water and hygiene practice properly [26].

As the present study attempted to collect appropriate primary data through observation and interview, the findings are highly valid and reliable for the study area and can be applied in other similar settings.

## Conclusions

This study found that significant proportions of households have no latrine and hand washing facility though they have access to improved water source. It was also found that only a few utilize latrine, hand washing, and drinking water properly. Further, though more than three-fourth (68.4%) of households had a latrine, the present study found that only 9.3% of them wash their hands after toilet. In general, our findings showed the availability and proper utilization of latrine, hand washing, and water depends mainly on the follow-up of households to encourage and educate on hygienic practices by either health extension workers, HDAs or the sanitation committee. To have important sustainable outcomes from CLTSH program, it was evidenced to provide training to local actors including HEWs and HDA [27]. Therefore, strengthening the HEWs and the HDAs to provide sustainable education and health information is needed.

## Abbreviations/acronyms

AOR: Adjusted Odds Ratio; CI: Confidence Interval; CLTSH: Community-led Total Sanitation and Hygiene; HDA: Health Development Army; HEWs: Health Extension Workers; HHs: Households; ICC: Intra-class Correlation Coefficient; PPS: Proportional to Population Size; HPs: Health Professionals.

## Acknowledgements

The authors are thankful for the data collectors and the study participants who involved in the study. We would like to extend our gratitude for College of Health Sciences, Mekelle University that supported on the overall research process.

## Conflict of interest statement

None declared.

## Authors' contributions

All authors involved in conception, design, coordination and supervision of the research project. AB, ZA and MB have made analysis of the data and interpretation of the results. AB and MB wrote the manuscript. All authors revised the manuscript and gave their contribution to improve the paper. All authors read and approved the final manuscript.

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■ Received on September 8, 2017. Accepted on June 19, 2018.

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ORIGINAL ARTICLE

# A study on the microbial quality of sealed products for feminine hygiene

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Keywords

Microbial flora • Safety • Tampons

Summary

**Introduction**. *Sanitary tampons have been in existence for over 60 years. Their use may present certain health risks, potentially associated with an abnormal change of microbial flora in the vagina (e.g., toxic shock syndrome). Tampon production and marketing are regulated differently in different countries. In Australia, Canada and the USA, tampons are classified as Class-II medical devices and their marketing requires pre-clinical and clinical studies, including microbiological trials. In Europe, tampons are considered consumer products and safety-related data are provided only if the manufacturer deems them to be useful. Sterility of these products is not requested by law; thus they may represent a potential vehicle for microorganisms. Due to the lack of data on microbial characteristics of tampons, an analytical investigation was carried out to characterize and quantify the microbial flora present on sealed tampons of various brands present on the market in Italy.*

**Methods**. *Traditional cultural methods were used to characterize and quantify bacteria and fungi. Identification of colonies was performed with biochemical techniques.*

**Results**. *Results showed low microbial concentrations in 93% of the positive samples. A rare presence of opportunistic pathogens was detected and a few samples (6%) were characterized by bacterial species of human origin.*

**Conclusions**. *In the light of these data, the examined tampons were found to have good hygienic quality. Nevertheless, to minimize the microbial risks linked to the use of these products, strict hygienic rules during their production and manipulation have to be adopted.*

## Introduction

The introduction on the international market of sanitary towels, also known as internal tampons, dates back to the thirties of the last century. The spread of tampon use, initially slowed by misinformation and some initial injuries, had a dramatic increase once its advantages over conventional sanitary tampons became apparent. The reassurance of freedom of movement in performing all types of work and sports during menstruation, combined with their protection and comfort are the characteristics that for over sixty years encouraged many women to use these products.

The function of the internal tampon is to absorb menstrual blood inside the vagina after it has left the uterus, preventing it from leaking out, and thus providing suitable protection with total discretion. The differences in the use of external tampons requires some knowledge of female genital tract anatomy for the tampon to be properly positioned, a prerequisite in ensuring total absorption. The choice available between the different tampon types with different absorption characteristics depends on the required level of protection, according to different needs.

Currently, internal tampons usually consist of an absorbent cellulose material and plastic derived material. They can be wrapped by a thin non-woven fabric layer and, at times, with an applicator in plastic or cardboard, and a cotton, polyethylene or viscose string for extraction [1]. These products are not requested to be sterile.

Over the years there were some problems related to the use of tampons, some, less serious, being vaginal dryness and ulcerations of the vagina, usually associated with the use of tampons with absorbency higher than the required needs [2]. Far more important however, the Toxic Shock Syndrome (TSS) can appear as a severe toxemia, sometimes rapidly becoming fatal. Its symptoms are high fever, vomiting, diarrhea, confusion and rash [3]. The disease is caused by the *Staphylococcus aureus* toxin 1 (TSST-1), bacterium that commonly colonizes nose and vagina [4]. The women most at risk of TSS are those with an earlier colonization of the vagina, regularly using tampons. It is likely that mechanical or chemical factors related to the use of tampons favor an increase of the bacterial toxin production which enters the bloodstream through the disruption of the mucosa [5]. The incidence of this disease in women has rapidly decreased following massive advertising campaigns on the role of the tampons and diaphragms, and after the withdrawal of some brands of tampons from the market [6]. According to recent estimates, the incidence of the disease is 3 cases per 10,000 menstruating women [7]. Moreover there is evidence that in some cases this also occurs in women not using tampons, and also in the post-operative

period and post-partum situations [8]. During use, the risk was related mainly to prolonged times of use (more than eight hours) [9]. Based on the results of some researches, an increase of glucose concentration, due to lysis of the carboxymethyl cellulose by the microbial flora of the vagina, can support the growth of *S. aureus* [10].
Regulations on production and marketing of tampons differ across countries, and are stricter in Canada, Australia and the United States [11, 12]. Internationally, there is no legislation providing for sterility requirements.
In the United States these products are classified as Class II medical devices. Special controls are required, and their put on the market is subject to the approval of the Food and Drug Administration [12]. In addition to requirements for shape, size, composition, absorption capacity and presence of chemical residues, the manufacturer must perform pre-clinical toxicological and microbiological tests. Particularly, for microbiological safety requirements, the manufacturer must demonstrate that the product does not favor the growth of *S. aureus*, does not stimulate the production of TSST-1 toxin, and does not alter the normal vaginal microbial flora. Clinical studies are also required if the products have new types of buffers, or when there are significant changes in the design and material used, compared to the traditional sold tampons [12].
In the European Union, tampons are not considered medical devices and therefore are not regulated as such. The producer can decide whether clinical trials are required to verify the safety of the product [13, 14]. In Italy, tampons, as other products for feminine hygiene, are consumer goods, and the safety obligations of the manufacturer and distributor are defined by the Consumer Code [15].
Given the mode of use, as well as providing a possible growth support for the microorganisms present on vaginal mucosa, tampons can be a vehicle of exogenous microorganisms in the female genital apparatus.
Numerous clinical studies have been performed to characterize the microbial vaginal flora during menstruation and to evaluate any change of its composition associated with the use of tampons [16, 17]. Many studies have also focused on the possible role of the chemical components of tampons as a potential support for microbial growth [10, 18]. Instead, currently, no data exist to estimate the possible impact in terms of the microbial concentration and type of species conveyed by non-sterile exogenous devices on the vagina.
Therefore an analytical investigation was performed to quantify and characterize the microbial flora present on tampons of various brands present on the Italian market.

## Methods

Fifteen brands of sealed internal tampons from five major manufacturers were subjected to microbiological analysis. The products were aseptically removed from their packaging, immersed in 100 mL of buffered saline solution added and stirred on a rotary plate for 2 minutes. The concentrated eluate was divided into several aliquots and the analyses were performed using the membrane filtration technique. Eight different groups of microorganisms/bacterial species were investigated. Thus, the membranes were incubated on various agarized media for the detection of the following microbial parameters:

- *Mesophilic bacteria.* Incubation on Plate Count Agar (Oxoid/ThermoFisher, USA) at 36°C for 72 h; the count of all colonies was made.
- *Fungi (molds) and yeasts.* Incubation on Sabouraud Destrose Agar (ThermoFisher Diagnostics, USA) at 25°C for 7-10 days; mold and yeast colonies were counted.
- *Anaerobic Bacteria.* Incubation on Plate Count Agar (ThermoFisher Diagnostics, USA) at 36°C in anaerobiosis for 72 h; the count of all colonies was made.
- *Coliforms.* Incubation on C-EC (Biolife, Italy) at 36°C for 24 h; blue colonies were counted.
- *Escherichia coli.* Incubation on C-EC (Biolife, Italy) at 36°C for 24 h; blue and fluorescent colonies were counted using a Wood lamp.
- *Staphylococcus spp.* Incubation on Baird Parker (ThermoFisher Diagnostics, USA) at 36°C for 48 h; the count of black colonies was made.
- *Candida albicans.* Incubation on Biggy Agar (ThermoFisher Diagnostics, USA) at 36°C for 18-72 h; dark brown colonies were counted.
- *Pseudomonas aeruginosa.* Incubation on Pseudomonas Agar/CN (ThermoFisher Diagnostics, USA) at 36°C for 48 h; the count of green-blue colonies was made, and a biochemical confirmation of fluorescent and reddish brown colonies using a Wood lamp was performed.

Biochemical identification: the grown bacterial colonies were isolated and identified by the miniaturized system VITEK® 2 Compact (Biomerieux, France).

## Results

From the 15 tampons examined, only one did not show microbial growth, while 93% of the samples exhibited a moderate bacterial load (Tab. I). A maximum of 100 cfu/tampon for the mesophilic bacteria and 55 cfu/tampon for anaerobic bacteria were counted, respectively. Molds were detected in 27% of the tampons, and the highest mold load did not exceed 15 cfu/tampon. Yeasts were absent in all samples. None of the examined tampons showed contamination by the fecal bacterial indicators, *E. coli* and Coliforms. The species *P. aeruginosa* and *S. aureus* were also absent in all samples, as well as *Candida albicans*.
From all the positive samples for the mesophilic bacteria, species belonging to the genus *Bacillus* were isolated. Bacteria of the genus *Alicyclobacillus* were also identified in one sample, and in 50% of the samples, *Staphylococcus epidermidis* and *Micrococcus luteus* were detected (Tab. II).

Tab. I. Results of the microbiological analysis of the tampons.

| Product code | Mesophilic bacteria CFU/ tampon | Molds CFU/ tampon | Anaerobic bacteria CFU/ tampon | *Staphylococcus* spp. CFU/ tampon |
|---|---|---|---|---|
| **1T** | 10 | < 1 | < 1 | 5 |
| **2T** | 20 | 5 | < 1 | < 1 |
| **3F** | 100 | 10 | < 1 | < 1 |
| **4N** | 100 | 5 | < 1 | < 1 |
| **5O** | 40 | 15 | 15 | < 1 |
| **6C** | 5 | < 1 | 55 | < 1 |
| **7C** | 20 | < 1 | < 1 | < 1 |
| **8O** | 10 | < 1 | < 1 | < 1 |
| **9T** | 10 | 5 | < 1 | < 1 |
| **10V** | 5 | < 1 | < 1 | < 1 |
| **11O** | 100 | < 1 | 5 | < 1 |
| **12T** | 10 | < 1 | < 1 | < 1 |
| **13L** | 100 | < 1 | < 1 | < 1 |
| **14T** | 5 | 10 | < 1 | < 1 |
| **15T** | < 1 | < 1 | < 1 | < 1 |

CFU: Colony Forming Unit

Tab. II. Bacterial species identified from the analysed tampons.

| Product code | Identified species |
|---|---|
| 1T | *Bacillus subtilis*<br>*Staphylococcus epidermidis* |
| 2T | *Bacillus subtilis*<br>*Bacillus licheniformis* |
| 3F | *Bacillus pumilus* |
| 4N | *Bacillus licheniformis*<br>*Bacillus subtilis* |
| 5O | *Alicyclobacillus acidoterrestris*<br>*Bacillus licheniformis*<br>*Bacillus subtilis* |
| 6C | *Bacillus subtilis* |
| 7C | *Bacillus circulans* |
| 8O | *Bacillus subtilis* |
| 9T | *Micrococcus luteus*<br>*Bacillus subtilis* |
| 11O | *Bacillus pumilus* |
| 12T | *Micrococcus luteus* |
| 13L | *Bacillus pumilus*<br>*Bacillus licheniformis* |
| 14T | *Bacillus circulans* |

## Discussion and conclusions

As well as other parts of the human body, the vagina is populated by numerous microorganisms which together constitute the vaginal microbiome. This ecosystem is typical model of well-organized balanced mutualistic consortium. The indigenous bacterial communities play a protective role in preventing colonization of host by opportunistic pathogens.

*Lactobacillus* is the dominant vaginal bacterial genus and, to a lesser extent, streptococci, enterobacteria, staphylococci, corynebacteria, anaerobic bacteria, *Gardnerella*, *Candida* and *Mycoplasma* even colonize this district. Some of these, whilst being potential pathogens, do not represent a real health risk, unless their concentrations increase in a non-proportional manner, attributable to imbalances due to various causes. Given the lack of sterility, potential inadequate measure of hygiene during the insertion, anatomical proximity of the genito-urinary system and intestinal apparatus, the use of internal tampons could be a potential vehicle for microorganisms.

However, the data obtained from the quantification and characterization of the microbial populations of these products showed not high concentrations of bacteria and molds.

As for the composition of the microbial flora present onto these products, the most common microorganisms were bacteria belonging to the genus *Bacillus*, known to be present ubiquitously in nature, to produce endospores, to be capable of withstanding particularly hostile environmental conditions. The isolated species from tampons, namely *B. subtilis*, *B. circulans*, *B. licheniformis* and *B. pumilus*, are not generally associated with pathological conditions, although the latter two have been reported as agents responsible for diseases in immunocompromised individuals [19].

*Staphylococcus* spp. and *Micrococcus* spp. were also isolated. Species belonging to these genera are common in human environments and on human body. In particular, *Staphylococcus epidermidis* is a member of the normal human cutaneous and mucosal flora and represents the 65-90% of all staphylococci that usually inhabit skin, vagina, urethra and mouth. In physiological conditions, the bacterium does not harm the host. However, in an impaired immune condition (undergoing surgical implants or transplants etc.), this species can become a commensal opportunistic pathogen, causing disease in immunocompromised and catheterized individuals [20]. Even *Micrococcus luteus* is part of the skin microbiome of mammals and is a ubiquitous species in the environment. It is not considered a pathogen, but an opportunist bacterium, responsible for skin infections, endocarditis, septicemia and septic shock [21].

Taking into account both the scattered detection of the bacterial species of human origin (6% of the samples) and their low microbial loads, the presence of these bacteria in the examined sealed internal tampons may not constitute a health risk to the consumer. Only in specific host immune debilitation conditions and at high concentrations these microorganisms can perform their pathogenic action. This aspect was taken into consideration when 1400 tampons were recalled because the company tests detected *Chronobacter sakazakii* on the plastic tubing [22]. In fact, this bacterium, responsible of vaginal and urinary tract infections, pelvic inflammatory diseases or other potentially life-threatening infections, represents a higher risk in immunosuppressed women.

In view of our results, for minimizing the risks of the microbial origin, next to strict hygienic rules during production and packaging of these products, the observance of good practices (e.g., hand washing) during their use

and manipulation has to be adopted for reducing potential allochthonous contamination.

## Acknowledgements

Funding sources: this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Conflict of interest statement

None declared.

## Authors' contributions

All authors discussed the data and results and contributed to the final manuscript.

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■ Received on February 23, 2018. Accepted on July 17, 2018.

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ORIGINAL ARTICLE

# Lifestyles and discomfort in a sample of young Romanian students

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Keywords

Lifestyle • Romanian young people • Substance abuse

Summary

**Introduction.** *The 40.3% of the Romanian youth population is at risk of poverty or social exclusion, and, in addition, the abuse of substances increases. It was interesting to evaluate the attitudes shared by pupils as well as their knowledge of these substances with a view to analyzing causes and types of risky behaviour in young people.*
**Methods.** *This is an observational study on the harassment influence in the lifestyle of a sample of Craiova high-school students The Fagerström Test was applied to evaluate the degree of nicotine dependence, and the data processed to assess the Odds Ratio.*
**Results.** *The sample was composed by 1,980 students with an average age of 17, of which 1,727 correctly responded to the questionnaire. The 37.4% of students admit to smoke, and the 67% to drink alcohol. The Fagerström test showed that the 68.2% of respondents are not highly addicted to smoke while the 31.8% was associated with a middle-to-high addiction level. A worrying weekly consumption of beer has been registered in the students. Regarding psychological distress caused by harassment a high Odds Ratio was recorded between the smokers and the drug users.*
**Conclusions.** *More than half of students did not know about the health consequences of smoking, and the same result was recorded about the consumption of alcohol. A special attention should be done to the understanding of harassment problem in the young people. In fact, almost all the respondents declared to have suffered abuse and admits to use drug and cigarettes.*

## Introduction

The collapse of the Romanian dictatorship in December 1989 resulted in the adoption of a series of political and economic innovations necessary to reach the standards required for entry into Europe along with the adoption of a Constitution in 1991, subsequently modified in 1993, a process which allowed the country to become a Parliamentary Republic. Before 1989, Romania had a Semashko-type healthcare model, but after the 1999 revolution it was replaced by the Social Insurance Healthcare Model (ASS) [1-3].
In 2016, the percentage of Romanians at risk of poverty and social exclusion was equal to 38.8%, while the poverty rate for the European Union was equal to 23.5%. With regard to gender, 18.4% of females were at risk of social exclusion (the European average is equal to 10.8%), while the percentage for males stands at 16.1% (the European average is 9.5%) and, furthermore, taking relative poverty levels into account, the population group found to be most at risk is aged 18 to 24 (30.2%) [4]. In Romania, in fact, more than a third of the youth population (40.3%) is at risk of poverty and/or social exclusion compared to Europe's 24.3% and statistics show an increase in obesity, sedentary lifestyles and the use of alcohol and cigarettes as well as an increase of drug use [5-11]. In recent years, more than 40% of health expenditure in Romania can be attributed to health consequences relating to behavioural risks, including drinking and smoking, a poor diet and lack of physical activity [12-14].
Preventive measures aiming to reduce social inequality and encourage the adoption of a healthy lifestyle is still the most efficient solution to discourage and combat these behaviours. In Romania, however, though measures have been undertaken to curb cigarette smoking, such as banning smoking in enclosed public places (2016) and the sale of tobacco-related products in all educational and health facilities, there is still no national strategy to curb alcohol consumption.
Alcohol prevention programs and educational campaigns run by NGOs (non-governmental organizations) include Alliance for Fighting Alcoholism and Addictions [15]. A report by the Romanian National Anti-Drug Agency shows that though their alcohol and cigarette consumption is higher, compared to young people in the rest of Europe Romanians are less involved in the use of illegal drugs [16, 17]. Drug use, however, has increased in recent years, though it is still lower than in other countries of the European Union. Cannabis consumption seems to have become common among young Romanians, as shown by the report by the European School Survey Project on Alcohol and Other Drugs [14]. The Agency points out that in Romania, the majority of drug addicts, mostly youths with no family or fixed abode, live in the slums of the capital city, Bucharest. In Romania, the

problem of child neglect was encouraged by a state law that until 2007 allowed the eradication of the child from the family of origin, if the parents did not guarantee the maintenance or care of the child [18].
In many cases, the psychological effects of the eradication causing by the traumatic event of the sexual and physical abuses (included beatings, suppression of meals, physical isolation, and submission to various humiliating jobs) by members of the staff and older children of the institutional care were exacerbated [19].
Considering the social-economic frailty of Romania's youth and the high percentage of psychological distress caused by harassments, it was interesting to evaluate the attitudes shared by pupils as well as their knowledge of abuse substances with a view to analysing causes and types of risky behaviour in young people [20].

## Methods

Research is an observational study performed following the Strobe-statement [21]. A questionnaire which was created with the aim of acquiring data and information on issues typically felt by young people, at 2016 to 2017. The only personal data it required was: gender, age, qualification and parental occupation. In order to be more easily understood, the questionnaire was written using colloquial terms which were closer to the mentality, habits and culture of respondents. Questions focused on the use of substances such as tobacco, drugs and alcoholic beverages, reasons for their consumption and knowledge of health consequences. The survey focused on a sample of Romanian high-school students in Craiova, a city in the region of Dolj in the South of Romania, with the aim of determining a group which was consistent with the diverse cultural, economic and social backgrounds in the territory. Part of the questionnaire focused on understanding bullying and harassment as experienced by young people, as this might increase the tendency to adopt risky lifestyles which are detrimental to health. The questionnaire, translated into the Romanian language, was initially validated on a sample of 100 high-school students to check whether questions were easily understood and easy to answer. The draft questionnaire, was given to a sample 100 high-school students, by trained professionals, to assess the face validity, and participants were asked to fill-in the questionnaire noting any questions unclear or ambiguous, and to provide any suggestions for improvement (ease of completion, legibility, time to fill-in etc.) [22, 23]. Subsequently, trained professionals distributed the questionnaire among six high-schools in Craiova. Criteria for selecting the high-schools were the presence in teaching staff of at least one teacher who was participating in a research project of Camerino University on harmful lifestyles in Italian and Romanian students, a problem that is deeply felt in Romania. Before compilation, each student was given the questionnaire in a closed, not glued envelope, which the student, after the compilation, sealed and placed in a box placed in a specific part of school. Compilation was autonomous and anonymous. Before the questionnaire was handed out, the professionals informed students on the aim of the survey, stressing its importance and utility. The study was conducted in accordance with the latest version of the Declaration of Helsinki, with the consent of the Institute Council of each selected school formed by the representative of the teaching staff and parents.
Data was promptly archived and elaborated using Access and Microsoft Excel. Statistical analysis was performed with X-Lstat software [24].
Descriptive statistics were used to analyze the distribution of variables. Qualitative data were described using frequencies and percentages. The Chi-square analysis and the Odds Ratio to evaluate the differences between smoking, alcohol, drugs consumption and abuse has been applied. The level of statistical significance was set at $p < 0.05$ with an confidence interval of 95%.
To assess the degree of nicotine dependence in current smokers, Fagerström Test for Nicotine Dependence (FTND) was used. Fagerström Test is composed of six items. In scoring the Fagerström Test for Nicotine Dependence, yes/no items are scored from 0 to 1 and multiple-choice items are scored from 0 to 3. The items are summed to yield a total score of 0-10. The global Fagerström score assesses the intensity of physical nicotine addiction: low dependence (0 to 2 scores), medium dependence (3 to 4 scores), high dependence (5 to 6 scores) and very high dependence (7 to 10 scores). A score of ≥ 6 has been used as cut-off to assess a high nicotine dependence [25].

## Results

Table I shows general characteristics in the sample of students who completed the questionnaire. A total of 1,980 students were contacted with an average age of 17, of which 1,727 correctly completed to the questionnaire (85.6%). The percentage of refusal adolescents was 14.6%.
Regarding the use of substances such as cigarettes, alcohol and drugs, the data shows 37.4% of students declear to smoking, starting when they were between 12 and 15 (41.3%) and in particular 42.5% are female. Regarding alcohol consumption, 67% make use of it and started drinking when they were 14 or 15 years old. On the contrary, 92% declare they do not use drugs.
A large part of participants were unable to answer the question on health risks regarding the use of amphetamine (49.8%), methadone (44.5%), LSD (43.1%), anabolic steroids (43.8%) and creatine (55.6%).
Answers show most students choose to abuse these substances mainly to "get high" (59.3%), in search of new sensations (25.2%), to feel good (19%) and to relax (18.8%).
The 18.2% of respondents reported that they use these substances to lose their inhibitions and of this sample, 8.9% to socialize more easily, 4.8% to show off and 4.5% to be accepted by their peers. It must be noted

**Tab. I.** General characteristics of the sample.

| General characteristics of the sample | n | % |
|---|---|---|
| **Gender** | | |
| males | 498 | 28.8 |
| females | 1,202 | 69.6 |
| no answer | 27 | 1.6 |
| Tot. | 1,727 | 100 |
| **Age** | | |
| ≤ 16 | 482 | 27.9 |
| 17-19 | 1,046 | 60.6 |
| ≥ 20 | 186 | 10.7 |
| Tot. | 1,727 | 100 |
| **Family origins** | | |
| Romanian: | | |
| mother | 1,619 | 93.7 |
| father | 1,621 | 93.8 |
| Other: | | |
| mother | 6 | 0.3 |
| father | 900 | 0.5 |
| No answer: | 199 | 11.5 |
| mother | 102 | 5.9 |
| father | 97 | 5.6 |
| **Parents' occupation** | | |
| Employed: | | |
| mother | 1,351 | 78.2 |
| father | 1,645 | 95.2 |
| Unemployed: | | |
| mother | 335 | 19.4 |
| father | 151 | 8.7 |
| No answer: | | |
| mother | 41 | 2.4 |
| father | 82 | 4.7 |

**Tab. II.** Fagerström Test for Nicotine Dependence (FTND) - scoring sheet.

| How soon after you wake up do you smoke your first cigarette? | | |
|---|---|---|
| | n | % |
| Within 5 minutes | 23 | 6.6 |
| 6-30 minutes | 46 | 13.2 |
| 31-60 minutes | 48 | 13.7 |
| After 60 minutes | 155 | 44.4 |
| **Do you find it difficult to refrain from smoking in places where it is forbidden (for example: in the movie theater, in the library, in church)?** | | |
| Yes | 288 | 85.5 |
| No | 43 | 12.3 |
| **Which cigarette would you hate most to give up?** | | |
| The first one in the morning | 210 | 57.5 |
| Any other | 155 | 42.5 |
| **How many cigarettes per day do you smoke?** | | |
| 10 or less | 189 | 54.1 |
| 11-20 | 77 | 22.1 |
| 21-30 | 17 | 4.8 |
| 31 or more | 5 | 1.4 |
| **Do you smoke more frequently during the first hours after waking than during the rest of the day?** | | |
| Yes | 46 | 13.2 |
| No | 256 | 73.3 |
| **Do you smoke if you are so ill that you are in bed most of the day?** | | |
| Yes | 92 | 24.5 |
| No | 283 | 75.5 |

that 20.9% of the general sample do it because they are bored.

In our evaluation of the extent of their reliance on these substances, two aspects deserve to be highlighted from the sample who smoke: 82.5% of respondents find it hard not to smoke in public places, though they are aware of passive smoking, with a difference between sexes (72.1% of females and 23.7% of males), only 0.6% of the total sample indicated cigarette smoke as being harmful to health.

Answers given to the Fagerström test (Tab. II) show that of respondents who smoke, 238 (equal to 68.2%) are not highly addicted to smoking, while 111 show middle-to-high addiction levels (31.8%). 40.4% of smokers do not believe they should stop smoking, though 77.1% of males declare their health is already suffering as a result.

Regarding alcohol consumption, data has evidenced a worrying weekly consumption of beer (81.3% of males and 66.5% of females) and a less frequent consumption of wine. The percentage of respondents who consume spirits is lower but still relevant, above all in males (40.8%) in comparison to females (28.6%). This type of drink is usually consumed during parties (62.7%), with friends (37.5%), at the disco (25.2%) and during holidays (18.6%). The 11.3% of students declare they also consume alcohol during school trips and 9.1% drink when home alone, too.

Regarding their knowledge of the health consequences of these habits, and more specifically of the dangers associated with cigarette smoke, 51.2% of all respondents did not answer, while 45% affirm they are conscious of the damage done by smoking, recognizing cancer as one of its principle consequences (24.7%), as well as lung cancer (16.8%) and other pathologies linked to the respiratory tract such as asthma, bronchitis and respiratory failure (13.4%). In addition, cardiovascular diseases (8.1%), TBC (5.5%), impotence and sterility (3.1%) were mentioned.

Regarding alcohol consumption, a high percentage of respondents (89.6%) are aware of the serious consequences of alcohol abuse, listing liver disease (38.6%), addiction (20.7%) and behavioural disorders, including violence and depression (10.3%). Other consequences are acute alcohol poisoning (6.2%), cardiovascular diseases (6.1%) and gastrointestinal disorders (5.9%). It also emerges that students are aware that alcohol may cause behavioural problems such as irritability, violent behaviour etc. (88.4%), cause car accidents (83.7%) as well as liver disease (79.6%). As regards their perception of the dangers of substance abuse, data shows that students consider drug abuse as the most dangerous activity (94.5%) while alcohol (64.7%) and smoking (64.3%) are considered less dangerous. In fact, when asked to indicate which substances are classed as drugs, only 33.7% selected alcohol and 24.2% smoking.

Regarding psychological distress caused by harassment, results showed that though only 12.8% declared they had been a victim, 35.1% did not answer the question and a further 2.6% answered only to rub out their answer. The remainder of the sample answered in the negative. The "special attention" received by respondents between the ages of 12 and 15 came mainly from friends or schoolmates (58.1%) but also from family members (7.2%), acquaintances (5.4%) and professors (2.7%). Answers to the question on whether this attention was perceived as having a sexual element showed that 36.5% did not think it was sexual, while 15.3% was explicit about its sexual nature, and 23.8% did not understand the real reason for this behaviour.

The students prefer not to describe their experiences in detail, and 10.4% declare either that they do not want to talk about it, do not remember what happened or that it was not important enough to tell (4.5%). 4.5% tell that they were touched in a sexual manner and 3.6% experienced verbal aggression.

An analysis of their reactions shows that they again prefer not to go into it (63.1% does not answer, 1.8% does not remember, 9.9% reacted with anger, 7.6% did not react and 5.8% tried to defend themselves). To the question "how did you feel about it?", 42.8% wanted to talk about it while 5% felt uncomfortable and afraid. To the question "in your opinion, is it useful to discuss harassment at school?" 45.9% thinks it would be useful to tackle this subject at school, to inform students (24.5%), talk about it (23.5%) and be prepared to deal with these events (21.6%).

A higher incidence of harmful lifestyles is noticed among young people who have suffered some form of abuse. In particular, these students smoke, use alcohol and above all drugs far more than those who have not been victims of abuse (Tab. III).

## Discussion and Conclusions

The data show respondents are suffering from a latent condition of unease. This pushes many of them, even adolescents, to choose harmful lifestyles.

The random sampling has been impossible to do for the bureaucracy of the Romanian administrative system, and then was difficult to extend the survey to all secondary school age groups, highlighting a higher percentage of female respondents with a prevalent age 17-19 years. In addition, another limitation was the impossibility to compare the sample interviewed with same young people Romanians not attending public schools.

Tab. III. Correlation between harmful lifestyles and abuse.

| Variables | Yes | No abuse | P value | OR | 95% CI |
|---|---|---|---|---|---|
| Drinker | 118 | 380 | 0.027 | 1.40 | 1.09 to 1.70 |
| Non drinker | 97 | 438 | 0.033 | 1.40 | 1.09 to 1.70 |
| Smoker | 146 | 486 | 0.002 | **1.63*** | 1.32 to 1.94 |
| Non smoker | 72 | 377 | 0.0055 | 1.57 | 1.26 to 1.88 |
| Drug user | 12 | 12 | 0.0003 | **4.00**** | **3.2 to 4.8** |
| No drugs | 204 | 815 | 0.0003 | 0.25 | 0 to 1.06 |

*: correlation significant of the P ($\alpha < 0.05$).

Young people are considered the most exposed to risks from harmful behaviour stemming from alcohol, tobacco and substance abuse [26, 27]. Starting to drink before the age of 14 can affect health negatively and is associated with alcohol abuse and other forms of abuse in adult life [28, 29].

In the sample, alcohol consumption is also present among high-school students (the youngest respondents). Over 70% consume alcoholic beverages, mostly beer, in line with figures reported by the World Health Organization in its 2014 Global Status Report on Alcohol relating to Romania [30]. The percentage of those who drink when they are home alone is worrying, as this shows young people are not able to overcome their feelings of distress. Concerning smoking, (aged 15) 37% of students have smoked at least once in the past week, while 17% smoke every day [31, 32]. Research has shown that 50% of young people have tried smoking at least once and a significant number (equal to 37.4%) have continued smoking. Their low perception of risk, typical of adolescence [33], means that the majority of respondents does not understand that substances which are seemingly less dangerous (like alcohol, cigarettes and cannabis) can become addictive and encourage pathologies in adult life. An analysis of behavioural differences based on gender shows that male adolescents are more superficial and tend to adopt harmful habits more easily (e.g., drinking alcohol, violence, tobacco use, alcohol and other drug use) [34, 35]. It must be said that the majority of respondents (75%) think that drugs are illegal substances, forgetting the effects alcohol and tobacco can have on the user, leading them to use them carelessly. They believe, in fact, that drug use is extremely dangerous, alcohol is less dangerous, and smoking is less dangerous still. Proof of this can be found in a survey conducted on young people in the European Union regarding the dangers of substance abuse, which shows that alcohol is seen as less dangerous (13%), as is smoking (14%) and cannabis (24%); on the contrary, the same youths regard heroin as being extremely dangerous (89%), together with cocaine (76%) [36]. In line with the European Commission's report, more than half of those who partook in the survey did not know about the health consequences of smoking, and did not see it as dangerous. In the same way, consuming alcoholic beverages seems to be seen in a similar manner, as safe [33]. Unsurprisingly, European youths classified as high-risk substances, if consumed regularly, cocaine (96%), followed by ecstasy (93%), cannabis (63%) and alcohol (53%); smoking was not mentioned [37]. On the contrary, regarding drug use, less than 5% of respondents takes illegal substances. This is confirmed by data from the surveillance project ESPAD in which the use of cannabis by Romanian students is equal to half of the ESPAD average (calculated with data obtained from 35 countries), with 2.7% of females and 4% of males. However, though Romanian adolescents use less cannabis, their consumption of new psychoactive substances (NPS) and other illegal substances is equal to, or slightly over, the average in other countries [16]. Even

though the number of those who declear to using illegal substances is low, their reasons for doing so are similar to the ones listed by young people in other countries: curiosity, seeking new sensations, to feel good, boredom, etc. [27, 38-41]. The questions on harassment deserve special attention, as this kind of abuse is surely more frequent than has been decleared; answers which were rubbed out and the lack of answers can be seen as confirming that some form of abuse has been experienced [42]. Among those who answered in the affirmative, it is worrying to see that some do not want to speak about it or remember it, showing the traumatic experience has probably not been faced. Lastly, considering that almost all respondents declared they do not use drugs, the ones who have suffered abuse and use drugs is up to 4 times higher than those who use drugs but have not suffered abuse. This increase can be seen for alcohol and smoking, too, though at a lower level. It seems urgent to improve efforts to educate about harmful lifestyles, not only simply by providing information on the effects of substance abuse or the legislation regulating its prohibition, but in the form of a constructive dialogue between professors and their charges, increasing communication and shared experience, in order to let adolescents choose healthy, satisfying practices, as called for by the students themselves.

Although surveys on young people and lifestyles have long been conducted at European level as well as surveys on the prevalence of child abuse in Eastern European countries, our research shows that, once again, an epidemiological investigation contributes to point out public health problems that then presuppose preventive medicine interventions. In our case it was highlighted a possible correlation between young people, who have suffered psychophysical abuse, and the use of substances of abuse such as drugs, alcohol and tobacco smoke. From what has been said, the need arises to investigate the presence of this hypothesis in a second study, relating the realities of other European countries.

## Acknowledgements

Funding sources: this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Conflict of interest statement

None declared.

## Author's contributions

FP conceived and coordinated the study, wrote the manuscript. SS wrote the manuscript. ET critically revised the manuscript. TTCN contributed to the acquisition of epidemiological data and statistical analysis. IG contributed substantially to the conception, design and supervision of the study

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■ Received on June 13, 2018. Accepted on September 3, 2018.

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ORIGINAL ARTICLE

# Fast food consumption and overweight/obesity prevalence in students and its association with general and abdominal obesity

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Keywords

Abdominal obesity • Fast foods • Iran • Overweight • Students

Summary

*Nowadays, the prevalence of both fast food consumption and overweight/obesity has been increased. This study aimed to estimate the prevalence of fast food consumption and to assess its association with abdominal and general obesity. In an analytical cross-sectional study, 300 students were selected randomly from two largest universities in Qom, center of Iran, studying in medical and basic sciences fields in 2015. Data collection was conducted by a modified version of NELSON's fast food questionnaire and anthropometric measures including Waist-Hip Ratio (WHR) and Body Mass Index (BMI). Chi-square, independent t-test, and multivariate logistic regression were used for statistical analysis. According to our results, 72.4% (67.4% in females vs 80.7% in males) had at least one type of fast food consumption in the recent month including sandwich 44.4%, pizza 39.7%, and fried chicken 13.8%, The obesity prevalence based on BMI and WHR was 21.3% (95% CI: 19.4, 23.2%) and 33.2% (95% CI: 0.7, 35.7), respectively. Fast food consumption was related to abdominal obesity as WHR (OR: 1.46, 95% CI: 1.11, 2.26), but was not related to general obesity as BMI (OR: 0.97, 95% CI: 0.63, 1.52). The prevalence of fast food consumption and obesity/overweight in Iranian student is high. Fast food consumption was associated with abdominal obesity based WHR, but did not related to general obesity based on BMI.*

## Highlights

In adolescent students, 72.4% and 34% have used at least one type of fast foods in recent month and in recent week.
The obesity prevalence based on BMI and WHR was 21.3 % (18.2% in females vs 26.3% in males) and 33.2% (40.1% in females vs 21.9% in males), respectively.
Fast food consumption was associated with WHR, while was not related to BMI.
Sandwich consumption was associated with obesity/overweight based on BMI to 35%, fried chicken to 40%, and pizza more than 80%.

## Introduction

The percentage of caloric intake from fast foods has increased fivefold over the past three decades among adolescents [1, 2]. In addition, obesity prevalence increased dramatically worldwide as one of the most serious public health problem especially in childhood and adolescents in current century [3]. Fast food consumption has increasing' trend due to convenience, costs, menu choices, flavor and taste [4]. About 30% of children to more than 50% in college students use fast food daily[2, 5]. Moreover, more than 33% of adults and 17% of children and teenagers are obese in united states [6]. Increased food consumption and substantial changes in the food habits are the most important factors of obesity epidemic [7] besides the poor diet among young people at recent years [8].
Wide ranges of causes are associated with obesity and overweight that varied from genetic to environmental factors [3, 7]. However, our surround environment is one of the key factors that effective in the rapid development of the obesity epidemic in the world [7]. Fast food consumption is strongly associated with weight gain and obesity. Fast food consumption could increase the risk of obesity and obesity-related diseases as a major public health issue [9, 10]. Obesity and overweight are the most important factors of non-communicable diseases related to years of life lost in cardiovascular diseases [11, 12].
Fast food is defined by a convenience food purchased in self-service or carry out eating venues without wait service [9]. Todays, the number of women in the workforce is increased due to changes in the family structure and urbanization in all countries over the past years. Moreover, the working of people for longer hours expands and the food and mealtimes have changed seri-

ously. A rapid growth is observed in fast food industries and restaurants [13]. Consequently, some worse consequences such as overweight and obesity have increasing trend [9]. Previous research has identified a strong positive association between the availability of fast food and its consumption as well as fast food consumption and obesity outcomes [5, 8, 10, 14, 15]. However, some studies assessed the fast food consumption on the general obesity based on Body Mass Index (BMI) [5, 8, 10, 16]. Nevertheless, the association between fast food consumption and obesity type (abdominal/general) is unclear [3, 10]. We aimed to estimate the prevalence of fast food consumption and obesity/overweight in two different governmental and nongovernmental universities, and to assess the association of fast food consumption with abdominal/general obesity.

## Methods

This cross-sectional study was conducted on 300 students of two large Universities in Qom, center of Iran, that randomly selected and studying in medical and basic sciences fields at spring 2015. Sample size was calculated based on the fast food prevalence in recent studies with considering the power equal to 90% and first type error equal 5% as well as based on the minimal significant difference expected regarding fast food consumption between the two university and students who used and not used fast food. The study subjects were selected based on the multistage sampling method. In the first phase, according to the stratified random sampling method, 150 students selected from the Qom Medical University, and 150 students selected from a nongovernmental University (Qom branch of Islamic Azad University). Then in each stratum, simple random sampling was used for selecting some classes and recruitment of students. In the third phase, in each selected class, all the eligible students were called to participate in the study. After describing the objectives and the method of data gathering, the informed consent is taken from all the volunteer subjects. Moreover, the ethic committee of Qom University of Medical Sciences approve the study protocol.

Data collection was conducted by a modified version of standard NELSON' fast food questionnaire [17]. The reliability and validity of this questionnaire is assessed by them and reported as a reliable measure with fair validity. Moreover, the content validity of modified version of questionnaire changed based on cultural and nutritional differences in Iranian people, was assessed by experts in epidemiology, nutrition and health education majors. Moreover, the reliability of questionnaire was assessed by Cronbakh Alpha and estimated as 0.861.

The main outcomes in our questionnaire were fast food consumption, type of fast food and the frequency of consumption. The variables that evaluated in fast food consumption were selected based on more frequent items that used in Iran based on cultural and religious condition such as different types of sandwich, fried chicken, fried potato, hotdog and pizza.

Obesity indexes data of such as waist and circumference for calculating Waist-Hip Ratio (WHR), height and weight for computing BMI were collected. Waist, hip circumference, and height of subjects were measured by anthropometric tape measure. Moreover, the weight of students was measured by a valid scale (SECA 830). BMI and WHR were calculated by standard formulae [18, 19].

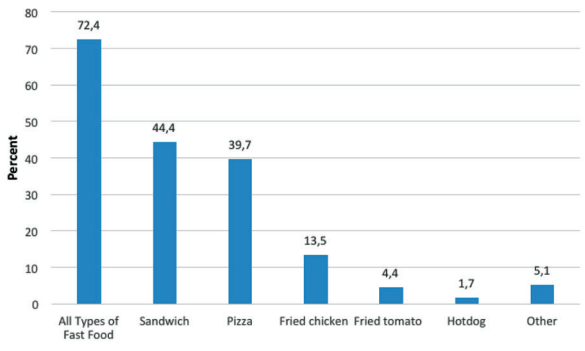
The WHR index was used for measuring the abdominal obesity and BMI for general obesity. Frequency, mean, and standard deviation were used for description of data. Chi-square test was used to assess the relationship between fast food consumption and quantitative demographic variables with obesity in studied subjects. Independent t-test were used for comparing the mean of age, BMI and WHR and their components in studied subjects between used and un-used fast food consumption. Finally, multivariate logistic regression was used to control the potential confounders including job, educational level, field of study and type of university. The statistical analysis was conducted using SPSS software (Chicago, IL, USA) and the type one error considered in 0.05 level.

## Results

Overall, 72.4% (67.4% in females vs 80.7% in males) have fast food consumption. These students used at least one type of the fast foods in the recent month. However, the most common type of fast food consumption was sandwich 44.4%, pizza 39.7%, fried chicken 13.8%, respectively. Figure 1 showed the distribution of different type of fast foods in recent month after survey.

Table I shows the comparison of fast food consumption in students by chi square test between who were consumed fast food in recent month and who not consumed. This table showed that there was significant difference between subjects who used and did not use fast food in recent month regarding to the gender, marital status, education level, university, and major of study. The married and male students as well as who studied in basic sciences and nongovernmental university were used more fast food. Nevertheless, there was no significant relationship between job and residency place at night with fast food consumption.

**Fig. 1.** The prevalence of different the types of fast food consumption in studied students.



Tab. I. The relationship between demographic variables and fast food consumption.

| | Used fast food | | Not-used fast food | | |
|---|---|---|---|---|---|
| Variables | N | % | N | % | P value |
| Gender | | | | | 0.008 |
| Female | 126 | 67.4 | 61 | 32.6 | |
| Male | 92 | 80.7 | 22 | 19.3 | |
| Marital status | | | | | 0.015 |
| Single | 176 | 69.8 | 76 | 30.2 | |
| Married | 42 | 85.7 | 7 | 14.3 | |
| Job | | | | | 0.547 |
| Student | 191 | 72.1 | 74 | 27.1 | |
| Employed | 24 | 66.6 | 10 | 34.4 | |
| Education level | | | | | 0.040 |
| BSc | 161 | 70.0 | 49 | 30.0 | |
| MSc | 56 | 81.0 | 13 | 19.0 | |
| University | | | | | 0.001 |
| Governmental | 94 | 62.3 | 57 | 37.7 | |
| Nongovernmental | 124 | 82.7 | 26 | 17.3 | |
| Field of education | | | | | 0.040 |
| Medical sciences | 161 | 70.0 | 69 | 30.0 | |
| Basic sciences | 57 | 81.4 | 13 | 18.6 | |
| Residency place at night | | | | | 0.632 |
| Own home | 41 | 78.8 | 11 | 21.2 | |
| Parent's home | 126 | 71.6 | 50 | 28.4 | |
| University dormitories | 49 | 70.0 | 21 | 30.0 | |

Table II shows that there was a significant difference between studied subjects who used and not used fast food in past month regarding to waist and WHR ($p < 0.05$). Nevertheless, the difference in age, weight, height, hip, and BMI was not significant between two groups.

The overweight/obesity prevalence based on BMI classification (higher 25 kg/m$^2$) was 21.3% (95% CI: 19.4, 23.2%) calculated 18.2% (95% CI: 16.1, 20.3) in females vs 26.3% (95% CI: 22.7, 29.8) in males. Moreover, the obesity prevalence based on WHR was 33.2% (95% CI: 30.7, 35.7) calculated 40.1% (95% CI: 36.6, 43.5) in females vs 21.9% (95% CI: 18.8, 25.0) in males, respectively. Therefore, we considered a subject as obese if he/she had BMI more than 25 or WHR more than 0.9 in males and more than 0.8 in females. According to this definition, 37.2% (41.2% in females vs 30.7% in males) were affected to overweight and obesity. Therefore, the consumption of fast food was related to obesity. Moreover, a significant relationship was observed between obesity and consumption of sandwich (OR: 1.35, 95% CI: 1.4, 2.41), fried chicken (OR: 1.4, 95% CI: 1.22,1.73), and pizza (OR: 1.8, 95% CI: 1.1, 2.9). In addition, the fast food consumption was related to WHR as abdominal obesity (OR: 1.46, 95 CI: 1.11, 2.26), but was not related to BMI as general obesity (OR: 0.97, 95% CI: 0.63, 1.52) (Tab. III). Based on multivariate regression model (Tab. IV) only marital status, type of university and gender were the most related factors of fast food consumption. Therefore, studying in nongovernmental university (OR: 3.16, 95% CI: 1.8, 5.6), single status (OR: 3.08, 95% CI: 1.26, 5.01) and being females (OR: 2.96, 95% CI: 1.61,4.53) are the most important related factors of fast food consumption, respectively in Qom, Iran.

Tab. II. Comparing the mean of age, BMI and WHR and their components in studied subjects between used and un-used fast food consumption.

| | Used fast food | | Not-used fast food | | |
|---|---|---|---|---|---|
| Variables | Mean | SD | Mean | SD | P value |
| Age (yr) | 21.37 | 2.20 | 21.52 | 2.40 | 0.619 |
| Weight (kg) | 64.20 | 11.30 | 61.90 | 11.2 | 0.130 |
| Height (cm) | 168.00 | 9.10 | 164.00 | 8.90 | 0.077 |
| Waist (cm) | 81.27 | 9.21 | 78.93 | 9.72 | 0.048 |
| Hip (cm) | 98.40 | 7.56 | 98.90 | 6.20 | 0.523 |
| Waist-hip ratio | 0.827 | 0.08 | 0.76 | 0.07 | 0.004 |
| Body mass index (kg/m$^2$) | 22.64 | 3.10 | 22.79 | 3.69 | 0.726 |

## Discussion

According to our results, 72.4% and 34% have used at least one type of the fast foods in recent month and recent week, respectively. It seems that the consumption of fast food in Qom students is high due to lack of recreational facilities and entertainment in this religious city. However, the fast food consumption in our study was lower than other studies [4, 20]. Results of studies in students of King Faisal University reported that more than 90% of people used fast foods monthly that was higher our estimate. In addition, a same study in female students aged 18 to 25 years showed that 47.1% had fast food consumption for two or more time per week [5].

The obesity prevalence in our study was estimated 21.3% and 33.2%, based on BMI and WHR, respectively. In a previous study, the obesity/overweight prevalence was 29.7% 5 and nearly half of them used fast foods. Moreover, in Shah et al. study, more than 34% of Chinese medical students were pre-obese and obese [4].

According to our results WHR was significantly different between subjects who used and not used fast food while, the difference in BMI was not significant. Therefore, fast food consumption was related to WHR, but did not related to BMI. In addition, consumption of sandwich, fried chicken and pizza were associated with obesity/overweight based BMI. Same direct association were demonstrated the association between fast food consumption and overweight/obesity in different studies [10, 14, 15, 21, 22]. Fast foods are poor in micronutrients, low in fiber, high energy density, high in glycemic load9 and large portion size with sugar [4] and could be more energetic than the daily energy requirements [6, 9]. In addition, the average energy density of an entire menu in fast food restaurant is approximately more than twice the energy density of a healthy menu [22]. According to some studies [3, 22, 23] obesity is the core of some important non-communicable diseases such as hypertension, hyperlipidemia, hypercholesterolemia, cardiovascular diseases, metabolic syndrome and type 2 diabetes [12, 22, 23]. Increase in energy density of diet by

Tab. III. The relationship between fast food consumption and obesity in studied subjects.

| Fast food consumption | Obese | Normal | OR (95% CI) |
|---|---|---|---|
| All type of fast food consumption | | | |
| No | 22 | 57 | 1.00 |
| Yes | 90 | 132 | 1.35 (1.41- 2.41) |
| Sandwich consumption | | | |
| No | 79 | 86 | 1.00 |
| Yes | 100 | 32 | 1.4 (1.22-1.73) |
| Fried chicken consumption | 87 | 169 | 1.00 |
| No | 24 | 17 | 2.74 (1.39-5.37) |
| Yes | | | |
| Fried potato consumption | | | |
| No | 107 | 177 | 1.00 |
| Yes | 4 | 9 | 0.735 (0.22-2.44) |
| Hotdog consumption | | | |
| No | 108 | 184 | 1.00 |
| Yes | 3 | 2 | 1.6 (0.78-3.37) |
| Pizza consumption | | | |
| No | 57 | 122 | 1.00 |
| Yes | 54 | 64 | 1.8 (1.13-2.90) |
| Obesity based on BMI | | | |
| No | 18 | 65 | 1.00 |
| Yes | 46 | 172 | 0.97 (0.63-1.52) |
| Obesity based on WHR | | | |
| No | 21 | 55 | 1.00 |
| Yes | 79 | 139 | 1.46 (1.11-2.26) |

Tab. IV. Multivariate analysis of predictive factors of fast food consumption in under studied subjects.

| Variables | Beta | SE of beta | P value | OR (95% CI) |
|---|---|---|---|---|
| Single marital status | 1.12 | 0.453 | 0.013 | 3.08 (1.26- 5.01) |
| Nongovernmental university | 1.15 | 0.228 | 0.001 | 3.16 (1.81-5.62) |
| Female gender | 1.08 | 0.312 | 0.001 | 2.96 (1.61-4.53) |

The adjusted variables in this model were job, educational level, field of study and type of university.

fat or sugar, together with concomitant eating behaviors like snacking, binge eating and eating out; promote unhealthy weight gain through passive overconsumption of energy [4, 6].

Fast food consumption is positively related to overweight and obesity due to extremely high energy density of these foods [6, 22]. Moreover, a study a significant association was observed between BMI and fast food consumption [4]. Two commonly eaten fast foods including fried foods and hotdogs have been associated with risk of obesity and weight gain [22]. Moreover, fast food consumption was related to general obesity in female adolescents. Moreover, obesity/overweight was significantly associated with frequency of fast food consumption [5].

This study found the prevalence of obesity was higher in females, while the prevalence of fast food consumption was higher in males. However, male students who are married are more interesting to eating fast food and it might be due to the religious culture of Qom as the most religious city of Iran. In the other hand, the single female students are not free to go in fast food restaurants than married ones. Moreover, three variables including marital status, type of university and gender are the most associated factors of fast food consumption. Based on our results in multivariate model, both studying in nongovernmental University and being single increase the odds of fast food consumption more than three fold. Moreover, female students used fast food 2.9 folds more than male students. The main reasons of students for fast food consumption are taste and comfort to access to these foods and lack of cooking skills [5]. The higher fast foods consumption in females and single students might related to lower wasting time in android social networks than male students [25, 26]. Moreover, since in nongovernmental university the price of kitchen food is high, the students are more interesting to have eating in fast food restaurants. However, the fast food prevalence is high in students and teenagers probably due to low cost [4, 16]. Nevertheless, because comfort accesses to fast food the corresponding expenditures are rising among people [15]. Moreover, the price of health outcomes of consequences of fast food consumption are more expensive and need to more investigations [9, 15]. We could not measure the morphometric characters and adipocity measures of students as other body compositions indexes. Moreover, lack of cooperation of students for anthropometric measurements was another limitation of the current study.

## Conclusions

The prevalence of fast food consumption and obesity/overweight in Iranian student is high. Studying in nongovernmental University, being single and females were associated with fast food consumption to three fold. Fast food consumption could have associated to abdominal obesity based WHR to 46%, but was not related to general obesity based on BMI. However, this study showed the different effect of fast foods on abdominal and general obesity as a hypothesis. Future studies need to determine the pure effect of fast food consumption on different dimensions of obesity.

## Acknowledgments

The authors would like to thank the research Vice-Chancellor of Qom University of Medical Sciences for financial supporting of this work. They are also grateful students who participated in this study.
Funding source: Qom University of Medical Sciences.

## Conflict of interest statement

None declared.

## Authors' contributions

AM: contributions to the conception, design of the work; analysis, and interpretation of data and Final approval of article.AA: contributions the acquisition and analysis of data for the work and Drafting the article. EM: contributions to the conception or design of the work; interpretation of data for the work; and Final approval of the article. SA: contributions to the conception or design of the work; interpretation of data for the work; and Final approval of the article. SK: contributions to the conception or design of the work analysis, or interpretation of data for the work; and Final approval of the article.HA: contributions to the conception, design of the work; analysis, and interpretation of data and Final approval of article.

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■ Received on September 18, 2017. Accepted on July 16, 2018.

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Overview

# The never-ending story of the fight against tuberculosis: from Koch's bacillus to global control programs

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Keywords

History of tuberculosis • Bacille Calmette Guerìn • *Mycobacterium tuberculosis* • Tuberculosis control strategy

Summary

*Tuberculosis (TB) is one of the oldest diseases known to affect humanity, and is still a major public health problem. It is caused by the bacillus Mycobacterium tuberculosis (MT), isolated in 1882 by Robert Koch. Until the 1950s, X rays were used as a cheap method of diagnostic screening together with the tuberculin skin sensitivity test. In the diagnosis and treatment of TB, an important role was also played by surgery. The late Nineteenth century saw the introduction of the tuberculosis sanatorium, which proved to be one of the first useful measures against TB. Subsequently, Albert Calmette and Camille Guérin used a non-virulent MT strain to produce a live attenuated vaccine. In the 1980s and 1990s, the incidence of tuberculosis surged as a major opportunistic infection in people with HIV infection and AIDS; for this reason, a combined strategy based on improving drug treatment, diagnostic instruments and prevention was needed.*

## Introduction and historical approach

Tuberculosis (TB) is one of the oldest diseases known to affect humanity [1]. It is caused by the bacillus Mycobacterium tuberculosis (MT): the bacilli usually multiply in the lungs and are spread by air-borne droplets from infected persons [2]. The disease reached epidemic proportions in Europe and North America during the Eighteenth and Nineteenth centuries [3] and today, with around 10.4 million new cases each year, almost one third of the world's population are carriers of the TB bacillus and at risk of developing active disease. India, Indonesia, China, Pakistan and the Philippines account for 56% of new tuberculosis cases [4], while the Democratic People's Republic of Korea, Lesotho, Mozambique, the Philippines and South Africa have the highest incidence rate, with over 500 new cases per 100,000 inhabitants [5]. In the past, MT displayed a predilection for young people and was called the "thief of youth"; in most cases, it proved fatal, with a death proportion in the pre-chemotherapy era: roughly 65% at 5 years, especially when it was associated to malnutrition, overcrowding and poor living and working conditions [6]. Over the last two centuries, the disease felled an estimated one billion people worldwide, and today, despite effective chemotherapy, it is estimated to be responsible for 1.7 million deaths, including the 380,000 among people with HIV infection normally classified under AIDS deaths by ICD convention, being the second greatest cause of mortality among infectious diseases in the world after human immunodeficiency virus (HIV) [1, 5]. Villemin was the first to prove that human tuberculosis was a contagious disease transmitted by an infectious agent, Mycobacterium tuberculosis [7]. This microbe was discovered and isolated in 1882 by the famous scientist Robert Koch, who was awarded the Nobel Prize for Medicine in 1905. After Villemin's definitive nosographic classification, the ravages of tuberculosis spurred many scientists in the following decades to seek an effective way of curbing the disease, and different and sometimes conflicting public health strategies were implemented [8].

## Isolation and staining of the Koch bacillus

Robert Koch observed that the alcohol-methylene blue staining developed by Karl Weigert in 1875 did not easily stain in tubercular lesions [9, 10]. He therefore implemented an innovative method of observing tubercular lesions by means of methylene-blue and a solidified medium of coagulated bovine serum heated to 40°C [11]. Using this staining technique, Koch reported that animal tissues and bacteria stained brown, whereas tubercular bacteria were easily detected on microscopy, as they appeared bright blue [12]. This innovation enabled Koch to prove that the various manifestations of tuberculosis, such as scrofula, pulmonary, extra-pulmonary and meningeal tubercular diseases, had a common cause, and also allowed him to reproduce the disease in experimentally inoculated guinea pigs [6, 11, 13-18].
Inspired by Koch's work, Paul Ehrlich further modified the staining protocol and began working on a better method of staining, using aniline water, fuschin and gentian-violet: the blue background counterstain showed

up the bacilli as red spots [18]. In 1882, Franz Ziehl introduced carbol fuschin instead of aniline, and Friedrich Neelson substituted sulphuric acid for nitric acid, which stained the bacillus a brighter red; this was known as the Ziehl-Neelson (ZN) stain or acidalcohol-fast bacillus (AAFB), since, once stained, the MT bacillus lipidic wall resists decoloring by acid and alcohol; for this reason, this technique is also used nowadays in order to isolate the MT bacillus [15]. In the following decades, microscopy continued to be the gold standard isolation method, and enabled a diagnosis to be made in a short time with high specificity. Moreover, it was cheap and simple to use [19]. In order to improve sensitivity, other methods of concentration, such as centrifugation, N-acetyl cysteine-sodium hydroxide, bleach, ammonium sulfate or chitin, were tested and used in countries where the disease displayed a high prevalence. In addition, the classical ZN staining was replaced by Kinvoun staining and immunofluorescence [19]. In more recent years, other cold staining methods were used to diagnose tuberculosis, such as Gabbet's method and a modified two-reagent cold staining method, which were less time consuming and easier to perform in the field [20]. Currently, the fluorescent stain auramine is used to better detect positive smears. In the past, its use was limited by the need to replace the light source in fluorescent microscopes every 200-300 hours. Subsequently, the innovative use of light-emitting diodes (LED), which last for tens of thousands of hours, proved to be a valid and more economical alternative to fluorescent microscopes, and today the World Health Organization (WHO) recommends replacing conventional ZN microscopy for the diagnosis of pulmonary tuberculosis even in resource-poor countries [21, 22].

## X-rays and the tuberculosis skin test: two effective diagnostic methods

Wilhelm Konrad von Röntgen was awarded the Nobel Prize for his contribution to the control of tuberculosis through his studies and his 1895 discovery of X-rays, which were used as an effective TB diagnostic technique. By using a Crooke's cathode, he succeeded in producing a shadow of a metal object on a photographic plate. This enabled him to visualize internal organs and detect TB lesions in the lungs, from Ghon foci to apical cavitation. This technique was used as a cheap method of diagnostic screening until the 1950s, together with the tuberculin skin sensitivity test. Nowadays X-rays are still used as a screening tool. In 1890, Koch obtained from tubercular bacilli a concentrated filtrate of liquid cultures, called "tuberculin" or "Koch's lymph" [23]. He believed it was an effective method of curing animals of tuberculosis, and announced his intention to test it in humans. Tuberculin was later nicknamed "la kockine", in French "coquine", which means "mischievous" as in "playful", but also as in "causing mischief" [7]. Koch had prepared the substance by means of crops in glycerin broth in six weeks, killed by heating at 100°C, filtered and evaporated to one tenth. Up to 0.25 ml of these crops could be injected into humans [24, 25]. It was a clear, brownish liquid, which was easy to store. However, it could be used in humans only in a very diluted form because it caused very violent reactions, such as inflammation at the site of inoculation, intense tuberculous reaction, high fever, expansion of tubercular lesions, more or less extensive necrosis and the onset of nephritis. This liquid was active only if injected subcutaneously, not when ingested. In healthy individuals, the subcutaneous injection of tuberculin did not cause any sort of reaction, while if injected into a subject already affected by the infection, it elicited an intense reaction, including fever, chills and vomiting, as happened to Koch himself [26, 27]. Tuberculin retained a place in the treatment of some forms of tuberculosis until the Second World War; later, it found application in the diagnosis of tuberculosis: the famous "tuberculin reaction" test, which used the same principle involved in the diagnosis of other diseases, such as glanders (Mallein Est) and brucellosis (brucella test) [24]. In 1907, a Viennese pediatrician and immunologist, Clemens Freiherr von Pirquet (1874-1929), used cutaneous tuberculin scratch tests, later called the Pirquet test, to diagnose in children "latent tuberculosis", a term which he himself introduced into medicine. The reaction to tuberculin, which he called "allergy", unmasked contagion even in individuals who showed no sign of the disease or who had overcome the clinically manifested disease [28]. In 1910, a French physician, Charles Mantoux (1877-1947), improved Pirquet's method, by using a cannulated needle and syringe to inject tuberculin between the layers of the skin. This permitted more accurate control of the dose of tuberculin used. Today, the gold standard test used to diagnose TB is tuberculin purified protein derivative (PPD). Developed in the 1930s by two American biochemists, Florence B. Seibert and Esmond R. Long, this is a more sensitive test than tuberculin [6, 28]. In 1952, Carroll Palmer and Leroy Bates published the results of a large study of reactions to one test unit of PPD-S in more than 3,000 hospitalized tuberculosis patients [29]. Fewer than one percent failed to react; skin reactions were normally about 15 mm in size. Some years later, other studies conducted in hospitalized tuberculosis patients and in school children in various parts of the world demonstrated that, in countries with a high prevalence of tuberculosis, a significant percentage of healthy school children displayed skin reactions that were similar in size to those seen in tuberculous patients [30]. This finding confirmed von Pirquet's theory of latent tuberculous infections, as did LB Edwards and CE Palmer's experiments, in which nonspecific tuberculin reactivity was caused by the cross-reaction to the antigen of environmental mycobacteria in guinea pigs [31]. Thus, tuberculin skin test studies contributed to our understanding of tuberculosis and other non tuberculous mycobacterial infections and of the immune response to human and animal TB [32].

## The role of surgery in treating tuberculosis: pneumothorax

In the diagnosis and treatment of tuberculosis, an important role was also played by surgery and other invasive techniques. Indeed, some anatomic and physiological conditions originally observed in patients with other diseases were successfully exploited to treat tuberculosis. The first such case was described in 1696 by Giorgio Baglivi, who reported that the clinical TB of a patient who suffered pneumothorax as a result of a sword wound improved. Later, in 1771, Edmond Claude Bourru, a librarian at the Faculté de Médecine in Paris, first suggested the benefit of lung collapse. Subsequently, in 1834 in London, FH Ramadge reported the first successful therapeutic pneumothorax in one of his patients. In order to cure and prevent the spread of TB infection, two surgeons, Edouard Bernard de Cérenville and Max Schede, used a new method of unilateral rib resection, called thoracoplasty, between 1885 and 1890. This enabled them to reduce thoracic cavity volume and collapse tuberculous cavities. In 1882, Carlo Forlanini, an Italian physician, undertook a new therapeutic intervention; he created the first artificial intrapleural pneumothorax by collapsing the lung and filling the pleural cavity with nitrogen. This technique at first appeared paradoxical, since spontaneous pneumothorax had, up to then, been seen as one of the complications of pulmonary tuberculosis. This therapeutic technique was successfully applied until the 1970s and was abandoned only after the advent of anti mycobacterial drug therapies [33, 34]. Thoracoplasty, which had first been introduced by the Swiss surgeon De Cérenville, was taken up by two Norwegian physicians, L Brauer and PL Fridrich, as a diagnostic technique for tuberculosis; its safety profile was very good as it did not involve the pleural space, and so minimized the risk of tuberculous empyema. Surgical treatments for tuberculosis were developed and used until the 1940s. However, they were followed by some complications, such as infections, fistula and empyema. The efficacy of these interventions was reported in various papers. For instance, in 1939 the Briton Oli Hjaltested published the results of a study conducted on 191 patients between 1925 and 1931 [35], while another paper described the outcome of 557 patients treated with pneumothorax between 1930 and 1939 [36]. After the discovery of effective antibiotic therapy, surgical treatments were progressively abandoned in favor of safer treatment. Neverless, there are some countries (e.g., former USSR) that kept using surgery abundantly under the claim that their cases are more advanced and complicated by drug resistance.

## Introduction of the tuberculosis sanatorium

The late 19th century saw the introduction of one of the first useful measures to combat tuberculosis, that is to say, improving hygiene conditions. Indeed, fresh air, proper nutrition and exercise had been prescribed for phthisis throughout history by famous scientists, including Hippocrates and Aretaeus of Cappadocia. In France, the government started to structure the fight against tuberculosis. "Selection centers" to identify soldiers with consumption, and "health stations", the precursors of "public sanatoriums", were created in order to treat tuberculosis sufferers, and above all to provide them with information on prevention, before they were sent home. Phthisiotherapy, also known as sanatorium treatment, was first proposed by a German physician, Hermann Brehmer. The sanatorium was a place where patients were treated with fresh air, rest, a rich diet, mountain walks and water [6]. In the middle of the Nineteenth century, the Italian doctor Biagio Castaldi, who suffered from pulmonary tuberculosis himself, published a summary of his clinical observations in the Journal of Medical Sardinian States [37]. He reaffirmed the great efficacy of a healthy lifestyle and a high mountain climate in preventing the disease. Indeed, he observed that these conditions could boost the body's mechanisms of defense and repair, first arresting the progression of the disease and subsequently healing local lesions and the general damage produced. At the end of the Nineteenth century, tuberculosis was widespread, especially among the poorer classes, and sanatoria were designed for these categories, too. In these places, people could work in order to obtain adequate treatment and food. Those who could not afford to stay in sanatoria were instructed to remain isolated from other persons living in the same house [13]. Treatment in sanatoria was undertaken in many countries until the Twentieth century, and several hospitals had therapeutic open-air wards. In most cases, however, only patients with minimal disease benefited; most of those with severe complications died within a short time [14].

## The prevention of tuberculosis: TB vaccines and Bacille Calmette-Guérin (BCG) immunization

In the 19th century, vaccination against tuberculosis was advocated in Italy by Edoardo Maragliano, followed by Giovanni and Gaetano Petragnani Salvioli, and in France by Albert Calmette and Camille Guérin. In 1886, the Venetian doctor Vittorio Cavagnis made the first pioneering experiment, in which killed bacilli were used in order to achieve a state of preventive immunity against infection. Cavagnis tentatively vaccinated some animals by means of the inoculation of tuberculous sputum treated with carbolic acid solution [38]. In 1891, Jacques-Joseph Grancher and P-Ledox Lebard experimented with immunization based on the principle of Pasteur's inoculations of crops of progressively increasing virulence. They started by inoculating avian tuberculosis bacilli cultures subjected to different aging processes, but had to admit that these cultures, when inoculated into guinea pigs, did not confer immunity against mammalian tuberculosis [39]. In 1895, Edoardo Maragliano claimed that it would always be preferable to use a vaccine with the bacillus of human type that contains the antigenic complex counterpart. He believed that the bodies

of desiccated bacilli were able to induce the production of specifically bactericidal substances in the organism into which they were inoculated [40]. On the basis of his research, he proposed a dual solution for the treatment of tuberculosis: an active specific therapy based on the administration of a tuberculin prepared by himself and a passive serum therapy, based on the administration of serum obtained from vaccinated animals. In 1901, Francisque Crotte proposed an "electrical" remedy for TB. A year later, von Behring, together with Romer and Ruppel, publicized a method called "jennerization" of cattle. They used a culture of tubercular bacilli of human origin, which had been maintained for over six years in the laboratory and then dried under vacuum, the virulence of which had proved extremely low in the guinea pig. Increasing amounts of the culture were injected intravenously into calves aged about six months on two occasions, initially with a 6-week interval, and then a 3-month interval, between injections. Another line of research was also moving forward; this was aimed at immunizing humans by administering live bacteria obtained from patients. Indeed, in France, Cavagnis had been injecting progressively higher doses of a mixture of phenolized water and saliva from tuberculosis patients. In 1905, two French bacteriologists and pasteurians, Albert Calmette and Camille Guérin, began their search for an anti-tuberculosis vaccine at the Pasteur Institute in Lille. On 8 January 1908, a strain of tuberculosis bacillus isolated by Nocard was cultured on pieces of potato cooked in 5% glycerinated beef bile; after 230 successive cultures, Calmette and Guérin observed in 1920 that these bacilli were no longer able to infect guinea pigs or rabbits, even at high doses [41]. The strain was called Billié Calmette-Guérin, from which they were able to produce a non-virulent strain which they formulated into a live attenuated vaccine, later called Bacille Calmette-Guérin or BCG [42]. In 1921, BCG was first administered to humans by two French physicians, Benjamin Weille-Hallé and Raymond Turpin, at the Charité Hospital in Paris. The vaccine was administered orally to an infant born to a mother who had died of tuberculosis shortly after giving birth; the child survived and did not contract the disease. The vaccine soon became popular throughout Europe and, over the next seven years, more than 100,000 children were immunized. Subsequently, the oral formulation of BCG was replaced by a subcutaneous formulation according to the method of Loeffler and Matsda (1913), in which the bacterial bodies killed by heat were first dried and then heated to 70°C for 15 days. The subcutaneous vaccine proved more active, but soon they noticed that it often resulted in considerable damage, causing the skin to ulcerate and even liquefy; they therefore adopted intradermal administration as the method of choice. A vaccine developed by Koch and named "Tauruman", made by using living human tuberculosis bacilli with attenuated virulence, was marketed in the early years of the 20th century. In 1930, 250 children immunized with a BCG vaccine in Lübeck in Germany were accidentally contaminated by virulent tubercular bacilli; seventy-three subjects died of tuberculosis in the first year and a further 135 were hospitalized [43]. The Second World War was followed by a resurgence of tuberculosis throughout Europe and Asia, and in 1948 UNICEF undertook a tuberculosis control program of tuberculin testing and BCG vaccination in children. Routine vaccination was discontinued in the 1970s and in the following decades, but is still implemented in many countries with a high prevalence of tuberculosis, in order to prevent childhood tuberculous meningitis and miliary disease, and in healthcare, military personnel and other people at high risk of exposure to tuberculosis.

## New prophylactic and therapeutic vaccines

Since the beginning of the 21st century, new prophylactic vaccines have been tested in order to better control the pulmonary form of TB. One strategy could be to boost the current BCG vaccine with new subunit vaccines. Moreover, more effective recombinant BCG or attenuated live vaccines have been tested for use in primary immunization [44]. In the past century, the BCG vaccine was used in various countries and protected many children and adults from TB. However, its effectiveness against pulmonary TB was variable. For this reason, various formulations of new-generation TB vaccines are currently being tested and are in various phases of clinical trials, the aim being to achieve lasting immunization [45]. In 2016, 13 candidate vaccines were tested in clinical trials, including candidates for the prevention of TB infection and candidates for the prevention of TB disease in people with Latent TB Infection [5]. These candidate vaccines are intended not only to provide stronger immunological responses against MT, but also to elicit long-lasting responses, which will require stimulation of memory T- and B-cell responses [46]. Currently, 8 of the 13 vaccines in clinical development are subunit vaccines; 6 of these contain or express either Ag85A or Ag85B proteins. A major challenge to TB vaccine development is the lack of diversity in both the antigens included in TB vaccines, and the immune responses elicited by TB vaccine candidates. Both will need to be expanded to maximize the potential for developing a successful candidate by 2025. [47] Live attenuated vaccines interfere with phagosome biology and host intracellular pathways, including apoptosis and autophagy. In recent studies, mucosal vaccination was found to be superior to parenteral vaccination, and this innovative route of administration is currently under study [48]. In recent years, a new hypothesis of cure was introduced by a new formulation of TB vaccine. The effectiveness of this vaccine is based on the theory that mycobacterial antigens can enhance bacterial killing. A phase II study of Mycobacterium indicus pranii (Mw) vaccine administered via the aereosol route is being examined in guinea pig and mouse models. RUTI is a non-live polyantigenic vaccine that may be used as a prophylactic vaccine together with short intensive antibiotic therapy; it is currently being tested in a phase II trial [5, 49].

## The discovery of streptomycin and other anti-tuberculosis drugs

During the 21st century, antibacterial chemotherapy was developed, with the discovery of numerous active molecules against the Koch bacillus, such as thiosemicarbazone, acid or para-PAS and hydrazide isonicotinic acid. In the 1930s, tuberculosis was shown to be resistant to sulphonamides and, in the 1940s, to penicillin. The Ukrainian microbiologist Selman Waksman, who was awarded the Nobel prize in 1952, coined the term "antibiotic". He first isolated actinomycin in 1940 and streptothricin in 1942, both of which would later be used effectively in TB therapy, but which were too toxic in their first formulations. Other anti-tuberculosis chemotherapeutic agents were developed in the following years, such as isoniazid, rifampicin, ethambutol and pyrazinamide, and more recently, viomycin and ciprofloxacin, used to treat drug-resistant infections. The strategy of early diagnosis and targeted therapy with chemotherapy is now considered to have the absolute best cost/benefit ratio.

## Tuberculosis and AIDS: the challenge of multidrug-resistant tuberculosis

In the 1980s and 1990s, the incidence of tuberculosis surged as a major opportunistic infection in people with HIV infection and AIDS, as a result of their immune system impairment. Today, TB is still a significant cause of both illness and death in developed countries, especially among immunosuppressed individuals; indeed, subjects with HIV have a higher TB-related mortality rate than the general population [50]. Moreover, the risk of developing tuberculosis is estimated to be between twelve-twenty times greater in people living with HIV than in those without HIV infection [5]. In 2015, 15% of tuberculosis patients worldwide (1.2 million people) had HIV co-infection, and in parts of sub-Saharan Africa, the figure was as high as 50-80%. Tuberculosis is the leading cause of death in people with HIV infection and AIDS; one in three AIDS sufferers dies from tuberculosis [51, 52]. While the incidence of HIV-related tuberculosis has declined in developed countries, owing to effective anti-TB and anti-HIV treatment, it remains high in many developing countries.[53] In a study published by Karo B et al. in 2017, HIV-infected patients who contracted TB showed redued CD4+ cell counts; in these patients, it is important to start adjunctive TB preventive therapy [54].
New strategies for reducing HIV-related TB infections could be: the use of cheaper, more effective and less toxic drugs; early diagnosis based on PCR technologies in the case of MDR-TB infection, as this may be cured with bedaquiline and delamanid, which are effective against both sensitive and resistant strains; and, in the meantime, improving the use of ART in HIV patients. In the near future, an integrated approach will have to be developed, including new and more sensitive diagnostic tests, together with rapid and effective therapy [55].

## Towards the elimination of tuberculosis: tuberculosis control strategy

In 1993, the WHO declared TB a global emergency, and in 1995 defined a tuberculosis control strategy – the directly observed treatment, short-course strategy, also known as DOTS – the most important health breakthrough of the decade in terms of the number of lives that could be saved [56, 57]. Thanks to the DOTS strategy, the global incidence of tuberculosis has fallen by 1.5% every year since 2000, and from 2000 to 2015 the mortality rate declined by about 22% [58]. Nevertheless, morbidity and mortality rates worldwide are still high, especially in developing countries; indeed, 60% of TB deaths occur in six countries: India, Indonesia, China, Nigeria, Pakistan and South Africa [50]. In 1998, the complete MT genome sequence was published, which raised expectations of developing better therapies for TB and vaccines to prevent it [59]. In recent decades, however, multidrug-resistant tuberculosis (MDR-TB) has emerged and is now present in most countries. Patients infected with strains resistant to isoniazide and rifampicin are incurable by means of first line therapy. The response to tuberculosis treatment may be affected by multiple factors associated with the host-pathogen interaction, including genetic factors and the nutritional status of the host [60]. Drug resistance surveillance data indicate that, in 2015, approximately 480,000 people contracted MDR-TB worldwide and 97,000 MDR-TB patients were started on treatment. Surveillance of TB drug resistance over the last two decades has informed and guided the response to the MDR-TB epidemic, and recent innovations in molecular diagnostics have prompted a definitive shift to routine surveillance [61]. In 2016, an MDR tuberculosis treatment regimen of less than a year's duration was recommended on a global scale. Five priority actions, from prevention to cure, are required. The issues of health system barriers, diagnostic and treatment challenges and inadequate funding for care and research must be urgently addressed [62]. Developing new drugs, improving diagnostics and introducing new TB vaccines are critical components of a strategy to combat TB in general, and drug-resistant TB in particular [63]. In TB endemic areas, children are particularly vulnerable to drug-resistant TB; for this reason, a major goal is to improve access to currently available vaccines and treatment, in order to curb the continuing spread of drug-resistant TB, especially in younger people. According to the WHO Stop TB program, daily treatment with isoniazid for subjects at high risk of active tuberculosis is an effective preventive measure at both the individual and collective levels. Other preventive measures are recommended in national programs and in the guidelines of the WHO stop TB program, the aim being to provide universal access to TB diagnosis and treatmen [64]. The discovery of Mycobacterium tuberculosis-specific immunodominant antigens has led to the development of interferon gamma-release assays, which have been shown to have high sensitivity and specificity for TB disease [65]. Despite considerable progress, however, these techniques should be improved

in order to extend their use to low-resource countries and to cases of paucibacillary TB [66, 67].

## Conclusions

In Western countries, tuberculosis has greatly diminished in recent decades, and in these areas vaccination remains a selective remedy for vulnerable groups. In the last decade, however, tuberculosis morbidity has increased, rising from three million to ten million new cases annually; this rise is associated with the growth of co-infection with AIDS, which occurs especially in developing countries, particularly in Africa. In addition, the problem of under-notification of cases of TB must be remembered, which will hamper future efforts to reduce the incidence of the disease. In highly endemic countries, the WHO continues to maintain mass vaccination to prevent childhood tuberculosis, though it is acknowledged that this cannot break the "chain" of disease, which is sustained by adults with full-blown pulmonary forms. Since 2012, the WHO has implemented the TB end strategy, which is based on the development of new tools to better detect, prevent and treat tuberculosis. In the coming years, the challenge will be to succeed in translating innovative discoveries into public health targets and socioeconomic interventions in local tuberculosis control programs.

## Acknowledgements

The Authors want to acknowledge Dott. Edoardo Maragliano.
Funding sources: this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Conflict of interest statement

None declared.

## Authors' contributions

MM and IB conceived the study, performed a search of the literature and they drafted and revised the manuscript. GB revised critically the manuscript. MM, GB and IB read and approved the last version of the manuscript.

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■ Received on July 29, 2018. Accepted on August 29, 2018.

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Errata corrige

# Errata

J PREV MED HYG 2018; 59: E128-E131

Original article

## Knowledge and attitudes on influenza vaccination among Italian physicians specialized in respiratory infections: an Italian Respiratory Society (SIP/IRS) web-based survey

A. GRAMEGNA[1], S. DELLAFIORE[1], M. CONTARINI[1], F. BLASI[1], S. ALIBERTI[1], R. TOSATTO[2], M. MANTERO[1]
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# Corrige

Original article

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