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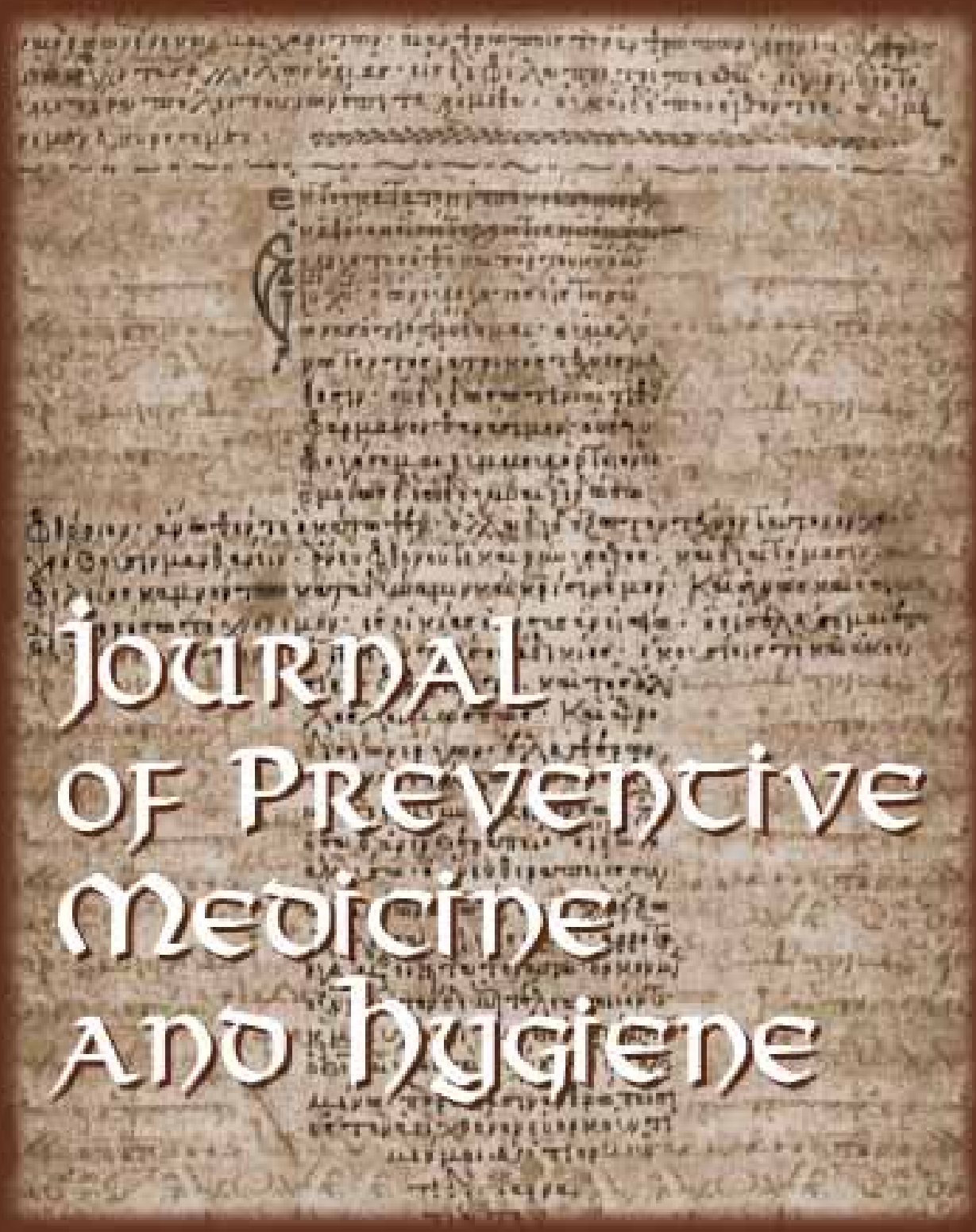

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CONTENTS

Overview

History and evolution of influenza control through vaccination: from the first monovalent vaccine to universal vaccines

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Keywords

Influenza • Vaccination • History of medicine

Summary

Influenza is a highly infectious airborne disease with an important epidemiological and societal burden; annual epidemics and pandemics have occurred since ancient times, causing tens of millions of deaths. A hundred years after this virus was first isolated, influenza vaccines are an important influenza prevention strategy and the preparations used display good safety and tolerability profiles. Innovative tools, such as recombinant technologies and intra-dermal devices, are currently being investigated in order to improve the immunological response. The recurring mutations of influenza strains has prompted the recent introduction of a quadrivalent inactivated vaccine. In the near future, scientific research will strive to produce a long-lasting universal vaccine containing an antigen that will offer protection against all influenza virus strains.

Introduction

Influenza viruses are negative-sense, single-stranded RNA viruses belonging to the *Orthomyxoviridae* family, together with Isavirus, Thogotovirus and Quaranjavirus. Three types of influenza viruses, namely influenza A, B and C, are capable of determining epidemics and pandemics in humans, with influenza A being the most common circulating type and causing significant illness, being most prone to antigenic shifts and the more likely type to lead to a pandemic [1, 2]. Recently, a new genus (termed influenza virus D) has been discovered in pigs and cattle with influenza-like illness syndrome in the United States [3, 4] and in Europe [5].

Influenza is a highly infectious airborne disease that affects a significant percentage of the world's population; local annual epidemics and pandemics have occurred since ancient times, causing tens of millions of deaths [6].

The aim of this mini-review is to provide a brief overview of the history and evolution of influenza and influenza control using vaccines.

A history of influenza: from the classical period to the nineteenth century

In 412 BC, in the "Book of Epidemics", Hippocrates described a putative influenza-like illness syndrome called "fever of Perinthus" or "cough of Perinthus" [7]. While some scholars claim that this is probably the first historical description of influenza (a winter and a spring epidemic of an upper respiratory tract infection occurring regularly every year at Perinthus, a port-town in Marmaraereglisi, a northern part of Greece, now Turkey), others, including the notable 19th-century editor of Hippocrates, Émile Littré (1801-1881), think that a diagnosis of diphtheria would better fit the description of complications (pneumonia, fits of coughing and wheezing, angina and paralysis of soft palate and limbs). On the other hand, symptoms such as disturbed vision and night blindness suggest a combination of diseases, including deficiency syndromes (e.g. vitamin A deficiency) [8]. In the years 1173 and 1500, two other influenza outbreaks were described, though in scant detail [9-11]. The name "influenza" originated in the 15th century in Italy, from an epidemic attributed to the "influence of the stars", which, according to Ginctrac, raged across Europe and perhaps in Asia and Africa [12].

It seems that influenza also reached the Americas. Scholars and historians debate whether influenza was already present in the New World or whether it was carried by contaminated pigs transported on ships. Some Aztec texts speak of a "pestilential catarrh" outbreak in 1450-1456 in an area now corresponding to Mexico, but these manuscripts are difficult to interpret correctly and this hypothesis seems controversial [13].

The first reliable documents regarding influenza-like ill-

ness syndrome date from 1510, when the virus spread from Africa to Europe. The first pandemic, or worldwide epidemic, occurred in 1557, though some scholars deny that it really was an outbreak of influenza. The first pandemic/worldwide epidemic that undoubtedly fits the description of influenza appeared in 1580, beginning in Asia and Russia and spreading to Europe via Asia Minor and North-West Africa. In Rome, it caused the death of over 8,000 people, while in Spain it decimated the populations of entire cities. Subsequently, it also affected the Americas [14].

Over the centuries, other pandemics were described worldwide. From 1404 to the middle of the 19th century, 31 influenza epidemics were recorded, including eight large-scale pandemics. Subsequently, others appeared, including three in the 20th century [14]. Some of the most notable outbreaks occurred in 1729, in 1781-1782 (a pandemic spreading from China to Russia, Europe and North America), in 1830-1833 (a pandemic which again spread from China to India, the Philippines, Indonesia, Russia, Europe and North America), in 1847-1848, and in 1898-1900 (spreading from Europe to India, Australia, and North and South America) [14].

One of the most devastating was the pandemic of "Spanish" influenza in 1918–1919, which caused an estimated 21 million deaths worldwide and was defined by Waring as "the greatest medical holocaust in history" [14, 15].

At the end of the 19th century, the etiology of this disease had yet not been well clarified; it was believed that the disease, termed "winter catarrh", was caused by bacteria (the so-called bacterial hypothesis), such as pneumococcus, streptococcus or *Haemophilus influenzae.* This latter was also named *Bacillus influenzae* or Pfeiffer's bacillus, after Richard Pfeiffer (1858-1945), who described it during the 1889-1892 influenza epidemic. This bacillus had already been discovered by the Polish microbiologist Bujwid Odo Feliks Kazimierz (1857-1942) in biopsy material a year earlier [16].

In the same period, the French microbiologists Charles Nicolle (1866-1936), Charles Lébally and René Dujarric de la Rivière (1885-1969) of the Pasteur Institute showed that the flu pathogen could pass through a fine filter. However, despite their brilliant experiments, the viral hypothesis continued to be neglected until the virus was isolated [16, 17].

In 1889, some Spanish doctors believed that influenza was a variant of dengue fever, whilst others attributed influenza outbreaks to a variety of causes including cannon fire on the western front, the building of the Madrid underground, air pollution, sunspots, or the spread of the habit of smoking poor-quality tobacco [18].

The thirties: virus isolation and the first experimental vaccines

During the 1918-1919 pandemic, some scientists began to suspect that bacteria were not the real agent of influenza disease. One of these was the scholar Richard Edwin Shope (1901-1966), who deeply investigated swine flu in 1920. However, it was only in 1932-1933 that the English scientists Wilson Smith (1897-1965), Sir Christopher Andrewes (1896-1988) and Sir Patrick Laidlaw (1881-1940), working at the Medical Research Council at Mill Hill, first isolated the influenza A virus from nasal secretions of infected patients, thereby demonstrating the intranasal human transmission of this virus [19, 20]. A few years later, the American virologist and epidemiologist Thomas Francis Junior (1900-1969) and Smith, in England, were able to transmit the virus to mice [21]. Subsequently, in 1935, Sir Frank Macfarlane Burnet (1899-1985) and Smith separately discovered that the flu virus could be grown on the chorio-allantoid membrane of embryonated hens' eggs [22], and in 1936 the first neutralized antibodies generated by infection by human influenza virus were isolated [23].

In the next five years, important developments took place: the demonstration that the virus inactivated by formalin was immunogenic in humans, purification of the virus by means of high-speed centrifugation, and the discovery that the influenza virus grew easily in fertilized hen eggs, a procedure that is still used today to manufacture most influenza vaccines [23].

The first clinical trials of influenza vaccines were conducted in the mid-1930s [24, 25].

A study by Smith, Andrewes and Stuart-Harris was conducted among military forces in England in 1937 using a subcutaneous vaccination with an inactivated strain isolated from a mouse lung [25].

In 1938, Francis, together with Jonas Edward Salk (1914-1995), managed to protect USA military forces. Salk would subsequently use this successful experience to develop an effective polio vaccine in 1952 [26, 27].

The forties: inactivated influenza vaccines

Influenza vaccination had two main objectives: (i) to protect against disease, and (ii) to achieve a high vaccination rate in order to ensure protection in unvaccinated people. The first vaccine was an inactivated, monovalent preparation which only contained a subtype of the influenza A virus [26, 27].

In December 1942, large studies were begun to be conducted on the first influenza virus vaccines; these provided the first official proof that inactivated influenza vaccines could yield effective protection against flu epidemics [28].

The efficacy and safety of inactivated vaccines were first studied between 1942 and 1945; in the meantime, a new strain of flu virus was discovered, the influenza virus type B, which is the main cause of seasonal epidemics, as was the phenomenon of so-called "influenza mismatch". Influenza mismatch is caused by major and minor mutations of circulating viruses. As a result, the virus contained in the vaccine does not match the circulating strain, determining a reduction in the effectiveness of subtype A influenza vaccines.

A new route of influenza immunization was tested in December 1942, with the subcutaneous inactivated bivalent vaccine containing viruses of type A and type B.

The following years, the first bivalent vaccine was licensed in the United States and became available for use in the general population [29, 30].

The fifties: influenza mismatch and influenza surveillance

The first system for the surveillance of circulating influenza virus strains in several countries worldwide was created in 1952 by the World Health Organization (WHO) in order to monitor the various virus mismatches reported. This important innovative tool enabled the composition of seasonal influenza vaccines to be determined on the basis of the epidemiology of influenza in the previous season [31]. In 1946, as a result of viral mutation, a new variant of influenza A (H1N1), A/FM/1/47, appeared in Australia. This gave rise to a new influenza subtype, the H2N2 strain, which caused the pandemic known as Asian flu [32].
The following year, the US Commission on Influenza recommended that a representative of this strain be included in subsequent vaccines.
The emergence of an HA subtype different from those circulating in previous seasons determined the need for pandemic influenza vaccines [31].

The sixties: split vaccines

New inactivated compounds were tested for safety and efficacy during seasonal epidemics in the 1960s, in particular two new formulations were created: split and subunit vaccines. The 1968 pandemic led to the development of trivalent inactivated vaccines (TIVs) against influenza viruses; moreover the development of new split or subunit vaccines led to a decrease of adverse reactions in children. These vaccines were split using ether and/or detergent, and haemagglutinin and neuraminidase were, in the case of subunit vaccines, purified and enriched [33].
In the same period, the first flu vaccines were licensed in Europe, while in the US annual influenza vaccination was recommended for individuals at major risk of influenza complications.
In 1968, the new virus strain H3N2 (Hong Kong) appeared, completely replacing the previous type A strain (H2N2, or Asian influenza), and led to another global pandemic with high morbidity and mortality [34]. In the same year, a new type of vaccine, the split vaccine, was authorized in the US after several clinical studies had demonstrated that it was less reactogenic than whole virus vaccines, especially in the early years of life [35].

The seventies: genetic reassortment

Split vaccines were widely used during the pandemic swine influenza in 1976 and in 1977, when the H1N1 subtype re-emerged worldwide. However, they were seen to be less immunogenic than whole virus vaccines in "primed" subjects who had never been vaccinated. Indeed, it was shown that two vaccine doses were needed in order to ensure effective protection [36].
At the beginning of the 1970s, an important innovation was introduced into the production of influenza vaccines: the genetic reassortment of influenza virus strains; this technique enabled the vaccine strains to grow faster in embryonated hen eggs [37].
The first subunit vaccine was created between 1976 and 1977. This contained only the surface antigens, hemagglutinin (HA) and neuraminidase (NA), which were isolated by means of successive purification steps.
This innovative tool proved to be highly immunogenic and well tolerated in humans, especially in children, although two doses were needed to guarantee vaccine effectiveness during epidemics [38].

The eighties: subunit vaccines

In 1980, the first subunit vaccines were licensed in the United Kingdom and are currently available in several countries worldwide.
In 1978, as a result of a major mutation, a new virus strain, H1N1, appeared on the global epidemiological scene. This strain, which was similar to a virus circulating in 1958, emerged in Russia and began to co-circulate, either simultaneously or alternately, with the previous one [39].
Antigenic drift, caused by frequent changes in the composition of the virus, determined the need to update the vaccine composition each year. This necessity prompted both the implementation of the first surveillance systems and the production of the first trivalent vaccine, which included three formulation strains (one strain of influenza A/ H1N1, an influenza virus A /H3N2 and a type B virus), in order to ensure effective protection during the 1978 pandemic.

Live attenuated influenza vaccines

In the period 1935-1941, the first clinical trials involving live attenuated influenza vaccines were conducted. The efficacy of these seasonal vaccines was guaranteed by the correspondence between the circulating strain and the strain contained in the vaccine and by the virus dose grown in hen egg embryos [34].
In 1944, Stanley described in detail the preparation and properties of an influenza virus vaccine produced in embryonated hen eggs; this vaccine was concentrated and purified by means of differential centrifugation and inactivated by means of various procedures [23].
In 1949, an important change in vaccine development involved the introduction of the use of cell cultures for virus growth.
In 1997,the so-called "avian flu" pandemic broke out in Hong Kong. This was caused by influenza virus A/ H5N1, a highly pathogenic strain.
In order to contain this pandemic, the techniques of genetic rearrangement developed in those years enabled a

huge number of vaccine doses to be produced in a short time by applying recombinant DNA technology to the influenza A/H5N1 virus [34].

Recent years

In recent years, scientific research developed new techniques of immunization, which may be more immunogenic and better tolerated during administration, thereby reducing adverse events. In 2003, for instance, the FDA in the United States authorized the use of an intranasally administered live attenuated vaccine, called FluMist®, in adults [40]. In the 2003-2004 influenza season, an outbreak in Asia was caused by an influenza A/H5N1 strain. This was later used to produce a vaccine, which was licensed in the United States by the FDA in 2007.
More recent years saw the development of adjuvanted vaccines, such as those containing alum adjuvants and the oil in water adjuvant MF-59, which significantly enhanced antigenicity [6].
Specifically, MF-59-adjuvanted vaccines were used in the elderly and in young children, and proved to elicit a good response even to pandemic strains with which subjects had not been primed by natural influenza infection. Similar responses were obtained through the use of other emulsions, such as stable emulsion (SE) and AS03, which were included in the 2009 pandemic influenza vaccines [36].
In the most recent pandemic season (2009), the influenza virus H1N1, which was transmitted to humans by pigs, was estimated to have caused more than 200,000 deaths in the first 12 months of its circulation [41].
A massive effort to produce vaccine for the new H1N1 strain began shortly after scientists identified the virus. The virus proved to grow slowly during the manufacturing process, which relies on cultivation of the virus in chicken eggs. Because of manufacturing delay, the vaccine was available in most countries after the second peak of influenza cases at the end of October leaving most people not immunized while influenza H1N1 virus was circulating [42].
In the elderly, the vaccine efficacy normally decreases, because of immunosenescence. For this reason, in 2009 the Advisory Committee on Immunization Practices (ACIP) recommended and authorized the use of high-dose Fluzone®, a new formulation containing a 4-fold higher HA dose than the traditional trivalent vaccine [43].
In 2011, as a result of developments in research into new vaccine delivery techniques, the FDA first authorized the intradermal administration of Fluzone®. This new route of administration involved antigen-presenting cells (APCs) in the dermis; these cells process antigens for subsequent presentation in the lymphoid organs, resulting in the stimulation of both innate and adaptive immunity. The intradermal vaccines elicited a better immunological response than intramuscular vaccines, particularly in the elderly; in healthy adults, it yielded an immune response comparable to that elicited by the traditional vaccines, while saving on the HA dose [44-48].
In 2012, the FDA approved Fluarix®, the first quadrivalent vaccine in the United States. This split vaccine contained two influenza A strains and two influenza B antigens. The presence of an additional influenza B strain reduced the possibility of a mismatch between the circulating viruses and the vaccine composition, while maintaining the same immunogenicity and safety as standard trivalent vaccines [49].
In 2013, the FDA approved FluBlock®, a recombinant trivalent influenza vaccine, for use in people aged between 18 and 49 years. FluBlock® was licensed in a spray formulation and was the first trivalent influenza vaccine made by using recombinant DNA technology. Derived from Baculovirus, it contained a 3-fold higher HA dose than traditional trivalent vaccines [50, 51]. The scale-up potential of the insect cell/baculovirus vector system may offer advantages in terms of rapid antigen change and response to a pandemic situation [31].
Currently, scientists are exploring the fascinating prospect of developing a universal vaccine by exploiting T-cells and by attempting to elicit broadly neutralizing antibodies. Moreover, efforts are being made to design M2e- or stalk-based vaccines, since these proteins (the type-2 matrix protein and the stalk domain of HA, respectively) are quite well conserved from an evolutionary standpoint [52, 53].

Conclusions

In the hundred years since the influenza virus was isolated, influenza vaccine preparations have evolved to ensure effective protection, while maintaining a good safety and tolerability profile.
The recurring mutations of influenza strains prompted the introduction of a quadrivalent inactivated vaccine, the composition of which is determined on the basis of the most frequent strains isolated in the previous season during continuous surveillance by the WHO.
Current research priorities include the development of a universal influenza vaccine that could offer protection against all influenza virus strains, thereby overcoming the challenges faced due to antigenic drift and shift or of co-circulation of different viral strains. Another important priority is to identify sustainable vaccine production platforms capable of rapidly meeting the large global demands for influenza vaccine in the face of an influenza pandemic.

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Authors' contribution

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

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ORIGINAL ARTICLE

Cross sectional study investigating the differences in knowledge and behaviors about HPV between vaccinated and non-vaccinated girls

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Keywords

HPV • Adolescent • Knowledge

Summary

Introduction. *The aim of the presents study was to compare the level of knowledge about Human Papilloma Virus (HPV) in vaccinated and non-vaccinated girls and to highlight the reasons why non-vaccinated girls refuse vaccination.*
Methods. *A cross-sectional study was conducted from October 2012 to June 2013 in Turin (Piemonte Region, Italy). Questionnaires were administered to girls attending secondary and high schools randomly selected.*
Results. *A total of 576 were compiled. The principle sources of information were parents and health workers. The main reported reasons for non-adherence to vaccination were the disagreement of the parents among the 11-12 years group (45.3%) and the lack of evidence on efficacy among the 18 years group (26.8%). By comparing the level of knowledge there was a statistically significant difference between groups: vaccinated girls reported higher score than the unvaccinated group in several questions ($p \leq 0.05$).*
Conclusions. *Our findings show a lack of information about HPV infection. Parents, school and health care workers have a central role in girl's education and choices about HPV vaccination. The communication campaign for the prevention of cervical cancer must therefore be characterised by messages able to clarify and consolidate messages that may have been partially received or misunderstood.*

Introduction

Cervical cancer is the first cancer recognised by the World Health Organisation (WHO) as entirely due to the Human papillomavirus (HPV) infection. The availability of two vaccines, along with the screening policies, allows an intervention for the prevention of cervical cancer [1]. In 2006, the WHO decided that, based on the available evidence, girls aged 9-13 years are the primary target of HPV vaccination [2-4], while the females aged 14-26 years are considered a secondary target.
The Italian health-care system is a regionally based national health service and each Region choses its vaccination strategy following the national recommendation that is to offer free HPV vaccination to 12-year-old females. However, some Regions offer vaccination to other age groups also, according to the WHO [1].
In particular, in Piemonte region (northwest of Italy), the vaccine is delivered by the Local Health Authorities (Azienda Sanitaria Locale, ASL) to 11-12 years old girls, and, in addition, from 2008 to 2011, 16-year-old female subjects represented the secondary target. The vaccination service calls each girl with a personal letter. The aim of the vaccination programme in Italy was to achieve coverage with three doses of vaccine equal to 95% within 5 years after the start of the immunisation programme.
However, both in Italy and other Countries, compliance were less than expected [3, 4]. The Department of Epidemiology of Infectious Diseases of the National Centre of Epidemiology Surveillance and Health Promotion (CNESPS) collects data twice per year regarding vaccination coverage according to Region, birth cohort and number of doses administered. For the birth cohorts in 1997-1998-1999, the document contains data with national coverage, updated on 30/06/2013, which amount to the following average values: 73% were vaccinated with at least 1 dose, 70% with at least 2 doses, and 67% with at least 3 doses [5]. In Piemonte region, the average coverage data for the three cohorts were slightly lower than the national average: 65.5% for the first dose, 66.8% for the second and 53.6% for the third dose. The vaccination rate for the secondary target does not differ greatly, reaching 61.6% in 2011 in the case of the Piedmont region, with different values across the ASL ranging from 46.4% to 75.7% [6]. Many studies have investigated the reasons associated with the low acceptance of HPV vaccination [7-13] such as: the availability of health care professionals to advise patients (the regarding to vaccination) [14], the will and the level of information of the parents [15, 16], the willingness of adolescents and young adults to receive the vaccination [17-19], and the awareness and knowledge of this infection [20, 21]. Some studies show low levels of knowledge about HPV and

cervical cancer in women, others indicate that knowledge among young male subjects is even lower [3, 22-24]. A low level of knowledge about HPV is also noticed among paediatricians and General Practitioner's (GPs) [25-28]. Studies show a significant desire for information [4, 18, 23], particularly among parents [20, 29, 30]. It is also demonstrated that where awareness campaigns are conducted the knowledge and attitudes of young people have improved [13, 31-35]. Several studies were carried out in Italy on this topic. Among Italian women, partial information is widespread regarding both HPV and vaccination [36]. The main source of information about HPV is represented by the mass media, while the role of health professionals is much less significant [37, 38]. Assessments about the reasons for this less-than-enthusiastic reception are in progress, in an attempt to identify the communication errors that might have caused it. There are still few studies on the target population [39-44]. It seemed useful, therefore, to focus attention on the information provided and the ability to properly train the adolescent population to highlight the possible need for additional information. The aim of this study is to investigate the knowledge about HPV infection and vaccination among girls aged 11-12 years and aged 18-19 years, highlighting the reasons that led to non-adherence to vaccination.

Methods

A cross-sectional study was conducted among a convenience sample of girls attending three secondary and two high schools in Turin hinterland.
The main reason for this choice is that schools are the suitable place to catch up both vaccinated and non-vaccinated girls. Questionnaires were realized based on literature information and tested in a small group of students (18 subjects). Subsequently, the questionnaires were administered in the schools by teachers assisted by several public health doctors between October 2012 and June 2013.
Subjects born in the years 1999-2000-2001, who were being convened at the time of the research, were chosen. The questionnaires were therefore distributed, with the same procedure mentioned above, to girls aged 18 who had been called for the vaccination when they were 15 years old as secondary target population.

The questionnaire

It was composed by multiple choices 11 questions for younger girls and by 18 questions for the older girls. The first part investigated the vaccination status (vaccinated vs. non-vaccinated), the presence and the nature of the sources of information. In the second part of the questionnaire, the knowledge about the disease and the possibility of its prevention, the acceptance of the immunisation, and the need for further informative opportunities were investigated. Furthermore we added more questions for the older girls regarding modes of transmission and HPV-related diseases. Each question had "yes", "no" and "don't know" as possible answer.
A written consent to fill in the questionnaires was asked for to underage girls' parents. We received the approval from the director and the internal committee of each school. Participation was voluntary, anonymous and without compensation.

Statistical analysis

Descriptive statistics were reported as frequencies and percentages for categorical variables. Comparisons of frequencies were performed using two-tailed chi-squared test and Fisher's exact test. Crude odds ratios (ORs) with 95% Confidence Intervals (95%CI) were calculated to assess the association between the level of knowledge in different groups. The data were processed using the StataMP13 statistical software (Stata Corp., College Station, TX, 2013).

Results

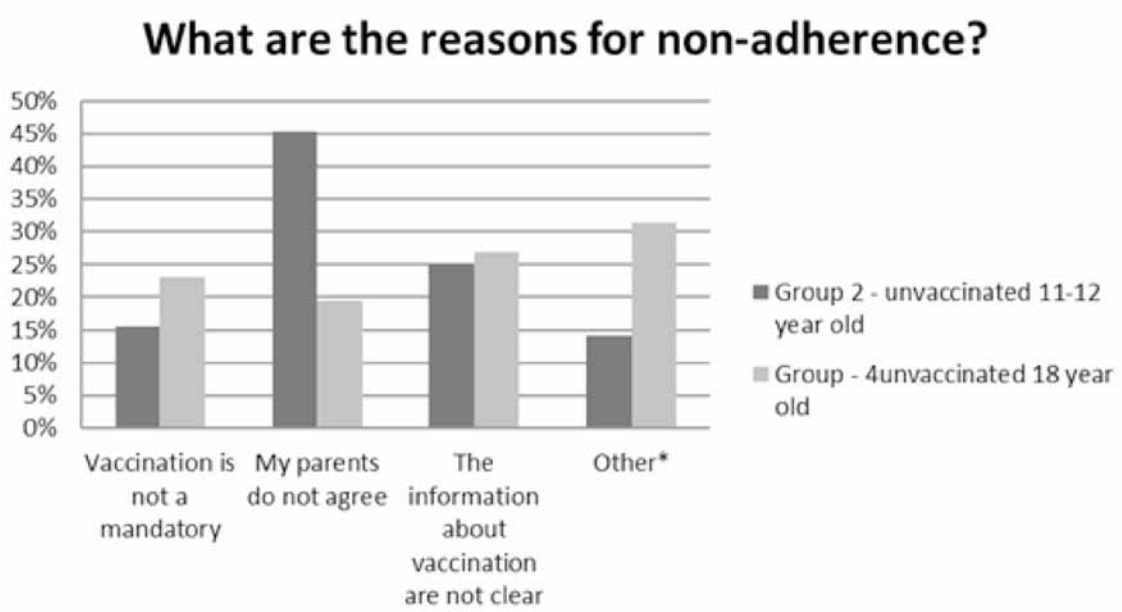
Sample characteristics

A total of 620 questionnaires were delivered to the girls, and, of these, 574 were returned (92.6%); 307 (53.6%) girls were unvaccinated, and 267 (46.4%) were vaccinated. Among these, 350 questionnaires were distributed in three middle schools, of which 327 were completed (93% of the sample); 128 girls had already been vaccinated (39% of the sample) (Group1), and 199 (60,8%) were not (Group 2); we included in this group, girls who were not already vaccinated, but called for the vaccination and inclined to do it (as indicated in the footstep note of the questionnaire by the girls); this is the reason why the coverage rate in this population appears lower than general population.
The other 270 questionnaires were distributed in two high schools, of which 247 (92% of the sample) were returned. A total of 139 girls were vaccinated (56% of the sample) (Group3), and 108 were not (43.7% of the sample) (Group 4).

Results of first part of the questionnaire

First part of the questionnaire showed that in Group 2, 76.8% of the girls stated that they have heard about HPV infection and know that a vaccine exists. In Group 4, 93.5% of the girls know HPV infection and 87% about HP vaccination.
We also asked to Group 2 and Group 4 what was the reason for non-adherence to vaccination (Fig. 1), in Group2 girls who refused the vaccination, the main reason was disagreement of parents (45.3%) followed by shortage of information (25%), the non-mandatory nature of the vaccine (15.6%) and other reasons (14.1%). In Group 4, the main reason for non-adherence was the lack of information about vaccines (26.8%) followed by the non-mandatory nature (23.1%) and the disagreement of parents (19.4%). Other reasons together represented 31.5% of answers.

Fig. 1. Reasons for non-adherence in group 2 and group 4.
* Other: the lack of confidence in a new vaccine, the vaccine does not protect against every type of HPV, fear of the still unknown side effects of this vaccine. The vaccine does not provide long-lasting coverage. The information received was not convincing. It has been discouraged by own doctor/gynaecologist. The vaccine is still experimental.



The questionnaire of the vaccinated girls (Group 1 and Group 3) investigated if they received explanation about HPV vaccination and from whom. In total, 82% of Group1 girls claimed to have received an explanation of the vaccine they had received during the visit and the reason why they were vaccinated, but 31% stated that they had yet to satisfy their curiosity about HPV infection and its vaccination. The majority of the girls claimed to have received information from their parents (70.3%); health care professionals of the Vaccination Service and paediatricians followed in a much smaller percentage, while gynaecologists, educators, the Internet, friends, advertising posters and flyers were marginal sources of information (Fig. 2).

In Group 3 72.2% of these girls received an explanation of what they received; 58% expressed some remaining curiosity. The majority of the girls claimed to have received information from their parents, their paediatrician/family doctor, and health care professionals of the Vaccination Service; a lower percentage (12-15%) received information from their friends, gynaecologist, the mass media, posters/flyers or the Internet or at school. Other sources were considered marginal (Fig. 2).

Results of the second part of the questionnaire

In the second part of the questionnaire, we investigated the level of knowledge about HPV infection and vaccination and then we compared the correct answers from vaccinated and non-vaccinated groups. This questionnaire part was constructed differently based on age, with more questions about transmission for the 18 years old group, for this reason we compared the groups divided for age.

Group 1: Already vaccinated 11 - 12 year olds, 31% answered "yes" when we asked if HPV

Infection is frequent and 23% stated that men could not be infected by HPV; only 38% knew that a person could be infected and aware of it. Data are presented in Table I.

Furthermore, 7% of the girls considered themselves to be at risk of contracting the infection in the future, and 38.2% did not answer the question; 77.3% stated that it would be useful to discuss these subjects at school.

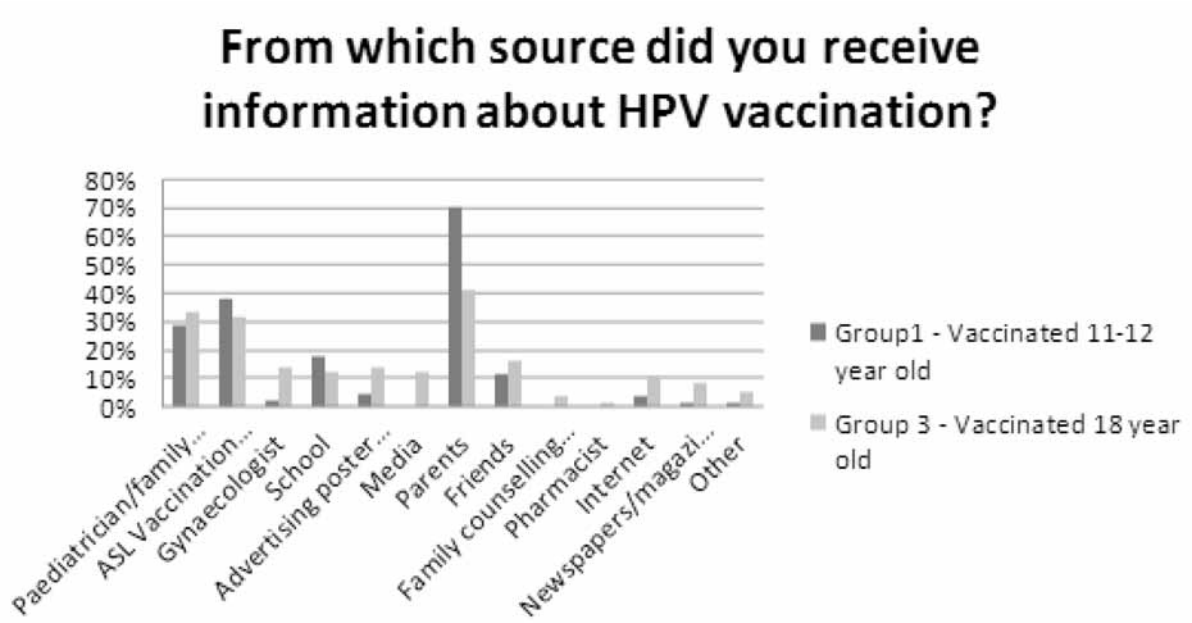
Group 2: 11-12 year olds unvaccinated due to rejection or waiting for the call, 65% of the girls stated they do not know whether HPV infection is frequent, 26% stated that men can be infected by HPV, and 42.2% knew that a person could be infected and be unaware of it. 12.5% of the girls believed they are at risk of becoming infected in the future, while 61.8% did not know the answer to the question; 71.8% stated that it would be useful to discuss these subjects at school.

The answers of these two groups are also compared in Table I, which shows that there were statistically significant differences between the frequencies of two of the statement: HPV infection is frequent (OR 1.75; CI(95%): 1.05-2.92) and HPV infection can cause cervical cancer (OR 2.65 CI(95%): 1.62-4.45), with higher correct response in the vaccinated group (Group 1).

Group 3: 18 years old, vaccinated

In total, 56% of the girls knew whether HPV infection is frequent, 93% did not know whether HPV caused genital warts, 27.8% did not know whether the male gender

Fig. 2. Sources of information in group 1 and group 3.



can be infected, and 47% thought that wearing a condom is sufficient to avoid contracting the infection. A percentage of 69% knew the mode of transmission of HPV, and 15% were not aware that HPV infection could cause cervical cancer. Table II summarises these data.

Group 4: 18 years old, non-vaccinated

In the non-vaccinated group, 24% of the sample knew the HPV infection is frequent, 7% knew that genital warts are caused by HPV, 21% did not know whether the male gender could be infected, and 26% said that wearing a condom is sufficient to avoid contracting the infection. A percentage of 47% knew that sexual intercourse are a possible way of HPV transmission, and 53% were aware that HPV infection could cause cervical cancer.

Comparing the answers from the two groups, as shown in table 2, a statistically significant difference was observed for the questions about the prevalence (OR 1.38 CI(95%): 1.04-2.92) and the consequences of the HPV infection (OR 1.10; CI(95%): 1.01-3.92); indicated that be vaccinated and receiving explanation about vaccination is related with higher knowledge about HPV.

Finally, the last question asked to the girls if they thought it would be useful to discuss these subjects at school, the majority of the girls (96%) answered yes to this question.

Discussion

The present study aimed to investigate the knowledge about HPV infection and vaccine among young girls in Italy (Piemonte Region). Interestingly, the answers to

Tab. I. Knowledge in Group 1 (11-12 years old vaccinated girls) and Group 2(11-12 years old unvaccinated girls) (the reference group OR = 1 is the one of unvaccinated girls).

	Correct answer			
	Group 1 N = 128)	Group 2 (N = 199)		OR CI(95%)
HPV infection is frequent	31%	21%	p = 0.03	OR =1.75 (1.05-2.92)
HPV infection can cause cervical cancer	76%	54%	p < 0.001	OR =2.69 (1.62-4.45)
Men cannot be infected by HPV	23%	26%	p = 0.654	OR =1.78 (0.52-1.49)
A person may be infected by HPV and not be aware of it	38%	42%	p = 0.5726	OR =0.87 (0.55-1.38)

Tab. II. Knowledge in Group 3 (18 years old vaccinated girls) and Group 4 (18 years old unvaccinated girls) (the reference group OR = 1 is the one of unvaccinated girls).

	Correct answer			
	Group 3 (N = 139)	Group 4 (N = 108)		OR CI(95%)
HPV infection is frequent	56%	24%	p = 0.001	OR = 1.38 (1.04-2.92)
HPV can be transmitted through sexual intercourse	69%	47%	P = 0.22	OR = 0.39 (0.81-2.37)
HPV infection can cause cervical cancer	85%	53%	p = 0.03	OR = 1.10 (1.02-3.92)
Genital warts are caused by HPV	7%	7%	p = 0.68	OR = 0.27 (0.4-4.0)
Men cannot be infected by HPV	28%	21%	P = 0.75	OR = 0.48 (0.52-1.60)
It's sufficient to wear a condom to avoid contracting the infection	47%	26%	P = 0.28	OR = 0.93 (0.78-2.22)
A person may be infected by HPV and not be aware of it	75%	51%	P = 0.37	OR = 0.29 (0.73-2.26)

the questionnaires showed some elements that can improve the efficacy of a vaccination campaign. The main source of information is represented by the parents, who, in most cases, are also those that bring girls to the vaccination centre; even in this case, the family is one of the most important source of information, in agreement with previous studies [8, 9, 30, 45]. Since the majority of the respondents recognizes the informational role of parents, it could be important to support and improve the knowledge of this source of information; conversely, health care workers appeared to have a slightly lower frequency as information providers and it could be of interest to understand why.

The majority of the girls stated that it would be useful to discuss HPV vaccination at school, that could play an important preventive role, by providing information about the different risky behaviours: in this sense, specific interventions may also be useful to clarify the contradictory messages that have spread, which have a negative impact on adherence to vaccination campaigns [23]. Interestingly, it should be stressed that in Countries such as the UK, an effective, comprehensive and organised school communication campaign has allowed us to reach coverage greater than 90% [23].

The majority of respondents claimed to have received explanations about the vaccination and its justifications; however, when knowledge about HPV infection and its mode of transmission were investigated, there was a statistically significant difference between the levels of knowledge of the two groups, which was higher in the group of vaccinated girls. The knowledge in the group of 18 year olds, questioned even on the "most sensitive" themes, showed a lack of information about the infection, its consequences, and modes of transmission and prevention; being vaccinated improve significantly the level of knowledge for few topics. It may therefore be important for health professionals and parents to be supported and to develop communication skills about sexuality [4, 17].

In the group of unvaccinated girls, both minors and adults, the lack of parental consent and of clarity of the information about the safety and efficacy of vaccination emerged as the main reasons behind this choice. One item worth noting is represented by the considerations of the involved teachers, who reported that the questionnaire was a first opportunity to address important issues to which little time and attention are usually devoted; at the same time the majority of the scholar said that could be interesting to discuss these topics at school; these findings are important to emphasize the role that school could have in sexual and health education.

Limitations of the study are the use of a convenience sampling, the peer influence of opinions between classmates and the issue of the reliability of the answers. Furthermore, the survey was conducted in the Torino area only, rather than opening up to different contexts, which could produce different results.

Conclusions

In conclusion, the proposed prevention model is based on a twofold assertion: first, the centrality of the adolescent and his/her action in his/her own health choices and, second, the strong educational role of the adult, the school and health care professional. The communication campaign for the prevention of cervical cancer must therefore be characterised by messages able to reach new cohorts every year in order to clarify and consolidate messages that may have been partially received or

misunderstood; particularly the information about long term efficacy, the appropriate age of administration and safety of both vaccines.

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Authors' contributions

CMZ conceived, designed and coordinated the research. MS and MVM collected data. MS, MVM and MRG performed the data quality control. MS and MVM optimized the informatics database. MS, MRG and MVM performed the statistical analyses and evaluated the results. MS 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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ORIGINAL ARTICLE

Molecular epidemiology and genotyping of *Chlamydia trachomatis* infection in a cohort of young asymptomatic sexually active women (18-25 years) in Milan, Italy

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Keywords

C. trachomatis infection • Sexually active asymptomatic young women • C. trachomatis genovars • Molecular epidemiology • Sexually transmitted diseases

Summary

Introduction. *Chlamydia trachomatis (Ct) is the most common bacterial cause of sexually transmitted infections (STI) and is associated with severe long-term sequelae in female populations.*
In Italy Ct infections are not submitted to a screening programme, and its epidemiological profile is understudied. Even scarcer information is available about the genetic diversity on ompA gene, whose sequence defines 18 different genovars. This study aims at evaluating the prevalence of Ct infection in young sexually active asymptomatic women aged 18-25, and characterizing the molecular epidemiology of the different circulating genovars in this population.
Methods. *Cervical samples collected from 909 sexually-active-young women (mean age 21.5 years) were analyzed through molecular assay for the detection of Ct infection. Phylogenetic analysis on the ompA gene was performed on Ct positive samples to identify the circulating genovars.*
Results. *The overall prevalence of Ct-infection was 4.4% (95%CI: 3.2-5.9%): 5.3% among women aged 18-21 years and 3.5% among those aged 22-25 years. Phylogenetic analysis has identified 5 different genovars: D, E, F, G, and H. The most common genovar was the E (46%), followed by genovar F and G (18.9% each), D (13.5%), and H (2.7%).*
Conclusions. *This study underlines the high prevalence of asymptomatic Ct-infections among young women. Overall, about half of the asymptomatic infections is sustained by genovar E. The introduction in Italy of a systematic screening program should be considered to allow a better understanding of Ct spreading and providing women with an opportunity for early treatment to protect their sexual and reproductive health.*

Introduction

Chlamydia trachomatis (Ct) is an obligate intracellular Gram-negative bacterium that is the most commonly reported microorganism responsible for sexually transmitted infections (STI) in Europe and is the cause of considerable acute morbidity and long term reproductive health problems, particularly in young people [1, 2]. Many infections are asymptomatic and result in delayed diagnosis and uninterrupted transmission. This is of particular clinical relevance as untreated infections can ascend in the female genital tract and cause Pelvic Inflammatory Disease (PID), which includes any combination of endometritis, salpingitis, tubo-ovarian abscess and pelvic peritonitis. PID can also result in ectopic pregnancy, infertility and chronic pelvic pain [2-4].
The presence of few or no specific symptoms that, if untreated, could lead to an increase in female reproductive tract morbidity and the existence of an inexpensive treatment against all these infections make chlamydia screening widely recommended to all sexually active women aged 25 or less [5-7]. In fact, Ct is under epidemiological surveillance in several European regions and the reported prevalence in young sexually active women ranges from 5% to 10%. Infection rates are the highest in females below 20 years of age and decrease with increasing age [8, 9]; in 2007, the incidence of chlamydial infection was 4.5-fold higher in the age group 15 to 24 years than in the age group 25-44 years [9].
In Europe the incidence of genital Ct infections seems to have increased dramatically over the last 20 years. However, this is most likely the result of more extended testing rather than a true rise in the incidence. Targeted screening, opportunistic testing for asymptomatic infections, contact tracing and mandatory notification help to explain the high notification rates in the UK and the Scandinavian countries compared with other European states [10].
In Italy, a reporting system for Chlamydia infections is not in place and an organised screening in specific asymptomatic population groups is not available. Surveillance data

are collected centrally by the Istituto Superiore di Sanità (ISS) by a sentinel network system consisting of 13 microbiology laboratories located throughout the Country. The last report, regarding data until 2012, described an overall prevalence of 2.4% in women aged between 15 and 45 years, with a significantly increased prevalence in lower age groups [11]. Moreover, a recent study identified 5.2% of Ct endocervical infection prevalence in a large population of sexually active women aged 15-55 years attending an outpatient service of cervico-vaginal pathology unit in Rome over a 10-year period [12]. Notwithstanding the existence of these studies, knowledge on the prevalence and molecular epidemiology of Chlamydial genital tract infections, particular in young asymptomatic women, remains modest.

Molecular epidemiology studies are currently based on the analysis of differences in the Ct major outer membrane protein (MOMP), whose coding gene (*ompA*) contains four spaced variable domains. Genetic variability on *ompA* gene reflects the existence of 18 genovars, classified according to their pathogenic potential [13]. Genovars A, B, Ba, and C have been commonly associated with trachoma, D-K, Da, Ia, and Ja with urogenital infections, and L1-L3 with lymphogranuloma venereum [13]. In addition, on the basis of amino acid similarities, these genovars have been grouped into the following groups or classes: group B (B, Ba, D, Da, E, L1, and L2), group C (A, C, H, I, Ia, J, Ja, K, and L3), and the intermediate group (F and G) [14]. These type of studies are useful to obtain new information about Ct genovars distribution, which can be further on translated into improved strategies for Ct infection management, for the traceability of sexual contacts, and in developing strategies for vaccine design [15, 16].

To improve our knowledge about Ct epidemiology and to expand the available Italian data, we evaluate here the prevalence of Ct infections and the molecular epidemiology of circulating genovars in young asymptomatic sexually active women aged 18-25 in Milan, Italy, over a period of 6 years (2008-2013).

Materials and methods

Study population

This was a cross-sectional study, performed on female subjects who spontaneously visited gynecological centers of the Local Health Units (LHUs) of Milan for medical consultations for contraception. No evidence of clinical symptoms related to Ct infection were described by the physician. Cervical cytological samples were collected for routine test among women aged 18 to 25 years, from September 2008 to June 2013. Written informed consent was obtained from young women so as to store their samples for further anonymous research testing. Due to its design, ethical approval was not required for this study, in compliance with the international policy [17] and with the current Italian legislation [18, 19]. The database was anonymised before the analysis.

Samples collection

Cervical cytological samples were collected using a brush (Cytobrush Plus Medscand® Medical AB, Sweden), immersed and rinsed in a vial filled with 20 ml of PreservCyt® Solution (PreservCyt) and stored at room temperature (RT) until processing. A total of 10 ml of each PreservCyt® Solution containing cervical cells was centrifuged at 3800xg for 15 min at RT. After centrifugation the pellet was re-suspended in 1 ml of Phosphate Buffered Saline (PBS), transferred in a new 1.5 ml collection test tube, and stored at -20°C until nucleic acids extraction.

DNA extraction and amplification

DNA was extracted with the EasyMAG kit (*NucliSENS® easyMAG®*, bioMérieux, France) from 500 µl of the resuspended pellet with a final elution volume of 100 µl. The concentration and purity of the extracted DNA was evaluated through a spectrophotometer (NanoDrop ND-2000/200C, Euroclone®, Thermo Scientific, Wilmington, DE, USA). After DNA extraction molecular tests were promptly performed to limit the storage to a maximum of a week. DNA integrity was assessed by the amplification of a 268 bp fragment of the ubiquitous beta-globin gene using the primer pair GH_{20} and PCO_4 [20].

A nested PCR targeting a Ct cryptic plasmid was performed to screen each extracted sample. The primers used for the nested PCR were previously described by Jalal et al. [21] and amplify a fragment of 150 nt. Amplifications were performed on 50 ng of extracted DNA in a 50 µlreaction mix containing 5x PCR Buffer, 200 µM dNTPs, 25 pmol of each primer, and 1 U Taq (GoTaq® DNA Polymerase 5U/µl Promega, Madison WI). Each run was accompanied by positive and negative control samples. The cycling conditions were as follow: 94°C for 5 min, followed by 30 cycles of amplification during the first step and 25 cycles during the nested step consisting of 94°C for 30 sec, 50°C for 30 sec, 72 °C for 30 sec, and a final 72°C for 7 minutes extension. The final amplification products were visualized using electrophoresis analysis on a 2% agarose gel containing ethidium bromide (0.5 mg/L) and compared with a standard (DNA Molecular Weight, Marker 100, SigmaAldrich, St. Louis, MO, USA).

All Ct-DNA positive samples were used to amplify a 395 bp fragment of the Ct *ompA* gene with previously described primer sets [21]. Amplifications were performed on 50 ng of extracted DNA in a 50 µl reaction mix containing 5x PCR Buffer, 200 µM dNTPs, 25 pmol of each primer, and 1 U Taq (GoTaq® DNA Polymerase 5U/µl Promega, Madison WI). The cycling conditions were: 94°C for 5 min, followed by 30 cycles during the first amplification round or 25 cycles during the second round of 94°C for 30 sec, 50°C for 30 sec, 72 °C for 30 sec, and a final 72°C step for 7 minutes.

Following the PCR of the *ompA* gene fragments, amplification products were purified using NucleoSpin® Extract II (Macherey-Nagel GmbH, Germany) and nucleotide sequences were obtained from automated DNA

Tab. I. Prevalence of *C. trachomatis* infections in different age groups of sexually active young women (18-25 years old).

Age group	Number of subjects N (%)	Ct-DNA + N (%)	Prevalence %	95% CI
18-19	220 (24.2%)	11 (27.5%)	5.0%	2.5-8.8
20-21	235 (25.9%)	13 (32.5%)	5.5%	3.0-9.3
22-23	230 (25.3%)	8 (20.0%)	3.5%	1.5-6.7
24-25	224 (24.6%)	8 (20.0%)	3.6%	1.6-6.9
Total	909 (100.0%)	40 (100.0%)	4.4%	3.2-5.9

sequencing on the ABI PRISM 3100 genetic analyzer (Applied Biosystem, CA, USA).

Phylogenetic analysis

Molecular characterization was performed by analysis of 395 bp amplicons of the *ompA* Ct gene (nucleotides 223-636 of Ct genovar A sequence, NC007429). All specimens presenting *ompA* variant sequences were confirmed by resequencing newly extracted DNA.

All sequences obtained in this study were deposited into NCBI GeneBank Database [27], under accession numbers: KX449367-KX449406. The reference genovars used for the construction of phylogenetic trees were obtained from the NCBI GenBank Database (*genovar* A: NC007429, M58938; *genovar* B: AF063194, U80075, M33636; *genovar* C: M17343; *genovar* D: NC000117, X62920, X62918, X62919; *genovar* E: X52557; *genovar* F: X52080; *genovar* G: AF063199; *genovar* H: X16007; *genovar* I: AF063200; *genovar* J: AF063202; *genovar* K: AF063204; *genovar* L1: M36533; *genovar* L2: M14738; *genovar* L3: X55700). Sequences were aligned using ClustalX 2.1 multiple aligner [22] and then used for phylogenetic inference. A model selection was performed to identify the best model for distance estimation. The evolutionary history was inferred using the maximum-likelihood method [23] based on the Tamura 3 parameter model [24], identified as the best fitting model after the model test analysis, using MEGA 6.06 [25]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (G = 0.8952) and phylogenetic trees were constructed with MEGA 6.06. A bootstrap test [26] with 1,000 replicates was performed to test the robustness of the analyses.

Statistical analysis

Data were expressed as mean (range) and percentages (95% confidence intervals, 95% CI) where appropriate. Comparisons between groups were performed using the chi-square test or Fisher's exact test. A p-value < 0.05 was considered statistically significant (two-tailed test). All statistical analyses were performed using OpenEPI software, version 2.3.1 [28].

Results

Prevalence of Ct infection

A total of 909 samples collected from the same number of sexually active young women (mean age 21.5 years, range 18-25 years) were available for this study. All women were asymptomatic for Ct infection.

The beta-globin gene was successfully amplified from all 909 cervical samples collected, confirming the suitability of the extraction method for these biological samples (extracted DNA: mean 20.1 ng/µl, range [3.3-45.7 ng/µl]).

The overall prevalence of Ct infections was 4.4% (95% CI, 3.2-5.9%). The prevalence of Ct infection was the highest among 20-21 year-old women with a value of 5.5% (95% CI, 3.0-9.3%) and decreased to 3.5% (95% CI, 1.5-6.7%) and 3.6% (95% CI, 1.6-6.9%) in age groups 22-23 and 24-25 years (Tab. I). Differences between infection rates in the different age groups and during the different years of the study period were not significant.

Molecular characterization

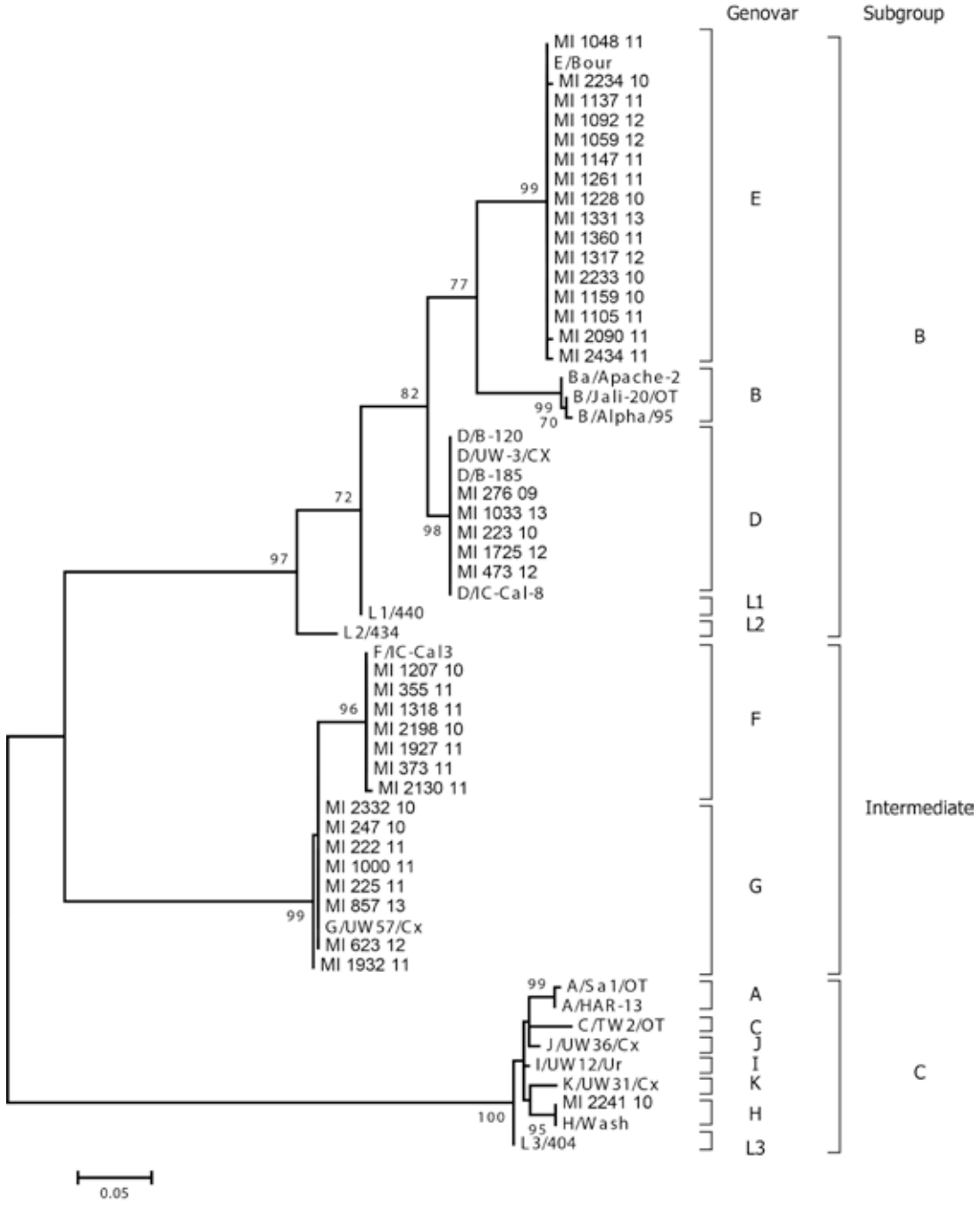
OmpA amplification was successful for 37 out of 40 positive samples (92.5%). Phylogenetic analysis of the *ompA* partial nucleotide sequences demonstrated 5 different genovars: D, E, F, G, and H (Fig. 1). In particular, 2 genovars (D and E) fell into subgroup B, 2 (F, and G) fell into the intermediate subgroup, and one, genovar H, fell into subgroup C. All circulating genovars are normally associated with infection of the urogenital tract.

The most common genovar in our population was E (46%, 17/37), followed by genovars F and G (both 18.9%, 7/37), D (13.5%, 5/37), and H (2.7%, 1/37). No genovar distribution pattern was observed among the various age groups (Fig. 2), and during the different years of the study period (Fig. 3).

Discussion

Chlamydia trachomatis is the most common bacterium associated with STIs and genovars D-K cause genital tract infections in women (cervicitis and urethritis) and men (urethritis). These can also be responsible for sexually transmitted rectal and pharyngeal infections, be transmitted during labor, cause pneumonia and eye infections in infants, and be spread by close contact to cause eye infections in adults. Several data suggest that, even though young people aged 15-24 years represent only 25% of the sexually experienced population, they acquire nearly half of all new STIs [29]. Rates of reported chlamydial infection among persons aged 15-19 years and 20-24 years continue to increase. Overall, during 2009-2010, rates increased up to 2.8% and 7.5% in the age groups 15-19 and 20-24 years, respectively [30]. Compared with older women, sexually active adoles-

Fig. 1. Phylogenetic analysis of *C. trachomatis* partial *ompA* gene sequences (coded between nt 223-636 of *C. trachomatis* genovar A, accession number NC007429) obtained during this study and compared to reference sequences. The outcome of the bootstrap analysis is shown next to the nodes, and branch lengths are proportional to genetic distances as indicated by the scale bar. Genovars as well as subgroups are indicated on the right.



cents aged 15-19 years and young adults aged 20-24 years are at higher risk of acquiring STIs for a combination of behavioural, biological, and cultural reasons [31].

This study focused on asymptomatic sexually active young women and describes the molecular epidemiology of Ct in Milan, Italy, over a period of 6 years. The prevalence of the infection was 4.4%, in line with what reported by other Italian studies [11, 12, 32, 33]. The most common genovars found in our populations were E (46%), F and G (both to 18.9%). Other recent studies described E (50-33%) and F (25-14%) as the most common genovars in symptomatic female populations [34-36]. A previous study, conducted in Italy in symptomatic and asymptomatic populations attending a Sexually Transmitted Disease (STD) outpatient clinic, described gerovar E as the most prevalent in female (37.2%), followed by genovar G (32.6%), and genovar F

Fig. 2. Genovar distribution among the studied age groups.

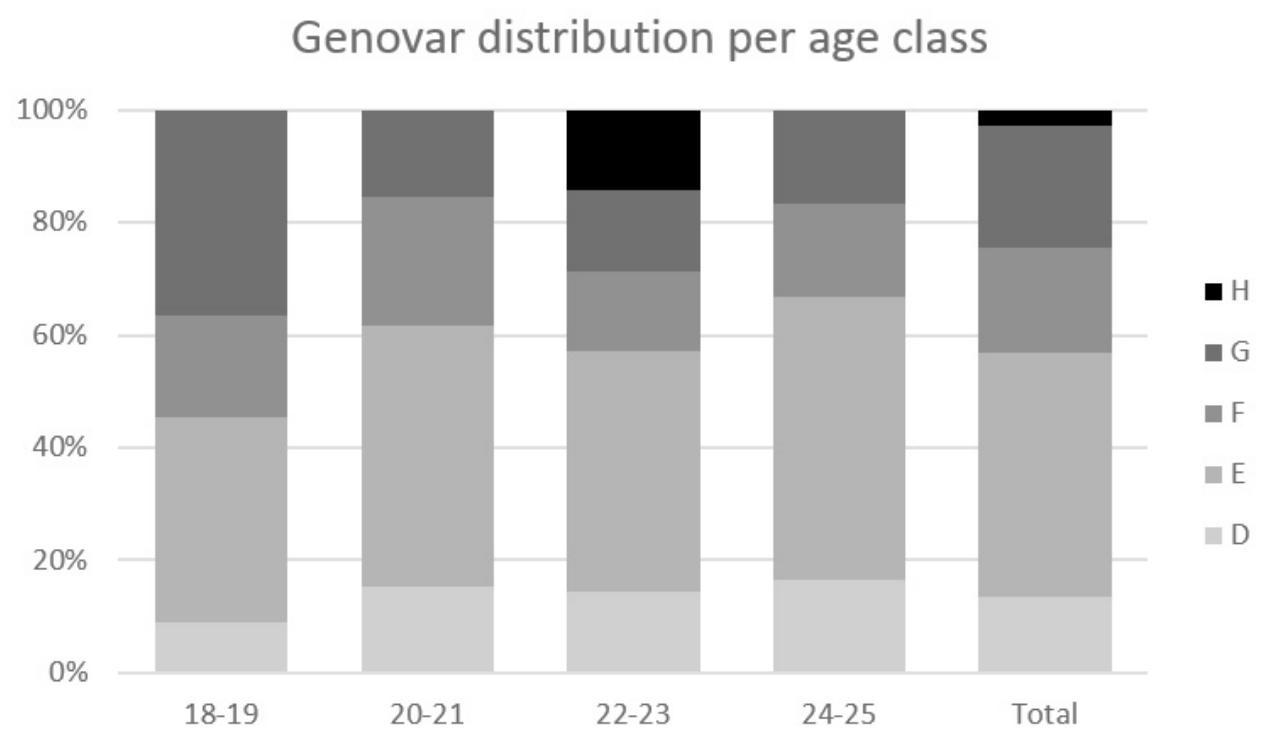
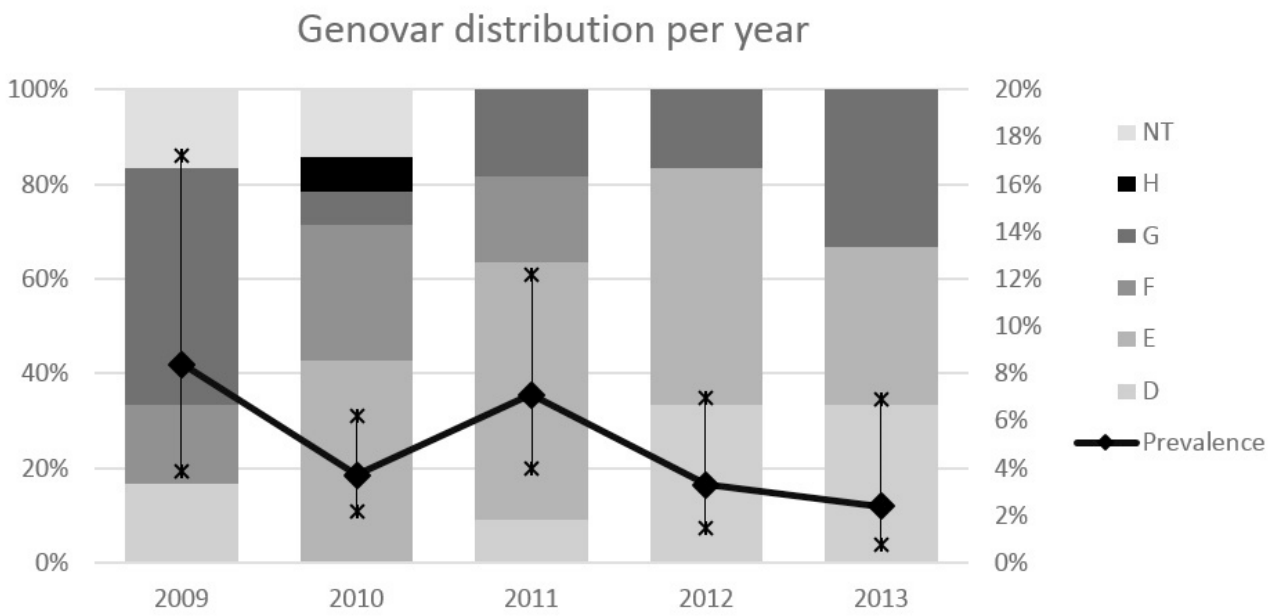


Fig. 3. Overall Ct infection prevalence (line) with 95% CI (vertical lines) and genovar distribution (bars) during the different years of the study. The vertical axis on the left refers to the genovar distribution, while the one on the right refers to prevalence values. NT: not typed.



and J (both to 7%), regardless of presence or absence of related symptoms [37].

It has been demonstrated that genovars E and F have a biological advantage over the other genovars thanks to both their ability to escape the host immune response and the presence of specific virulence factors, which can facilitate the transmission and infectious processes [38, 39]. It is possible that genovars E and F are less immunogenic than other genovars and, therefore, remain the most prevalent strain in all populations,

regardless of the presence or absence of clinical signs. Although we could not detect any relationship between infection, related genovar, and absence of symptoms, the present study contributed to increase the current knowledge on genotype distribution of Ct in asymptomatic young women in northern Italy.

Conclusions

Our data indicate that Ct infections occur frequently in young sexually active women and that several different genovars are widely spread in Italy. Larger national and longitudinal studies are definitively required to better understand the spread of Ct infection and its impact on the young Italian population.
These results underscore the need to establish primary and secondary preventive measures and to allocate more resources for an adequate prevention of Ct infections. The introduction of an opportunistic screening program for Ct in Italy should be evaluated in order to achieve early treatment of infected subjects and reduce associated clinical manifestations. Finally, it seems essential to develop educational programs and information campaigns, particularly addressed to young people, about acquisition, risk factors and treatment of STIs with a particular focus on *C. trachomatis* infection.
The Authors have no conflict of interest to declare.

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Authors' contributions

SB: study design, project and protocol development, data analysis and manuscript writing. ERF: project and protocol development, phylogenetic analysis and manuscript writing. MC: phylogenetic analysis and manuscript writing. DC: sample and data collection, protocol development and critic revision of the manuscript. EF: laboratory testing for *C. trachomatis* DNA and critic revision of the manuscript. AA: study design, supervision of the study and manuscript writing. ET: conception, design, coordination and supervision of the study, manuscript writing.

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ORIGINAL ARTICLE

Temporal trends of healthcare associated infections and antimicrobial use in 2011-2013, observed with annual point prevalence surveys in Ferrara University Hospital, Italy

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Keywords

Healthcare-associated infections • Antimicrobial use • Point prevalence surveys

Summary

Introduction. *Healthcare associated infections (HAIs) and misuse of antimicrobials (AMs) represent a growing public health problem. The Point Prevalence Surveys (PPSs) find available information to be used for specific targeted interventions and evaluate their effects. The objective of this study was to estimate the prevalence of HAIs and AM use, to describe types of infections, causative pathogens and to compare data collected through three PPSs in Ferrara University Hospital (FUH), repeated in 3 different years (2011-2013). The population-based sample consists of all patients admitted to every acute care and rehabilitation Department in a single day.*
Methods. *ECDC Protocol and Form for PPS of HAI and AM use, Version 4.2, July 2011. Risk factor analysis was performed using logistic regression.*
Results. *1,239 patients were observed. Overall, HAI prevalence was 9.6%; prevalence was higher in Intensive Care Units; urinary tract infections were the most common HAIs in all 3 surveys; E.coli was the most common pathogen; AM use prevalence was 51.1%; AMs most frequently administered were fluoroquinolones, combinations of penicillins and third-generation cephalosporins. According to the regression model, urinary catheter (OR: 2.5) and invasive respiratory device (OR: 2.3) are significantly associated risk factors for HAIs ($p < 0.05$).*
Conclusions. *PPSs are a sensitive and effective method of analysis. Yearly repetition is a useful way to maintain focus on the topic of HAIs and AM use, highlighting how changes in practices impact on the outcome of care and providing useful information to implement intervention programs targeted on specific issues.*

Introduction

Healthcare associated infections (HAIs) represent a growing public health problem in terms of patient safety and economic burden [1-3]. The Center for Disease Control (CDC) estimates the increased mean length of hospital stay for each HAI to be 7 extra days, ranging from 1-4 days for urinary tract infections (UTIs) to 7-30 days for pneumonia (PN). In Europe, HAIs cause 16 million additional days of hospitalization per year, 37,000 related deaths and 7 billion euros of additional costs (direct costs only) [4]. The Italian National Health Institute estimates 450,000-700,000 HAIs per year in Italian hospitals, 30% of which could be prevented; HAIs could be directly responsible for 1,350-2,100 avoidable deaths per year [5]. Misuse of antimicrobials (AMs) is a growing public health problem worldwide, associated with an increase in drug resistant microorganisms and adverse drug reactions that generate huge economic costs [6, 7].

The implementation of surveillance systems for both HAI and AM use is a relevant topic in modern public health [8, 9]. Although continuous surveillance still represents the gold standard for infection control, it requires a huge amount of human and economic resources but has rarely been used in multicenter studies. Instead, Point Prevalence Surveys (PPS), despite their inherent limitations in terms of accuracy of results and possibility of bias, are a highly feasible alternative, easier to perform even on large scale multicenter studies, less expensive and less time consuming. PPSs offer many benefits, including easy repeatability and the ability to provide meaningful information to be used for specific targeted interventions. The introduction of standardized protocols such as the European Center for Disease Control (ECDC) Protocol for PPS of HAI and AM use in acute care hospitals, version 4.2 2011-2012 [10], guarantees consistency of results and easy repeatability. Results of local surveys may also be used for yearly intra-hospital

comparison or benchmarking at regional, national or international level. In Ferrara University Hospital (FUH), infection and AM stewardship by PPS began in 1992, with a local Protocol and data entry form, updated over the years in agreement with the literature references [11]. This Protocol was used until 2011, when FUH participated in the first full scale ECDC PPS, October 2011.
The survey was repeated in 2012 and 2013. Objectives of these studies were: to estimate the overall burden of HAIs and use of AMs in the FUH; to describe HAIs and AM use by type of functionally homogeneous wards; to allow a comparison of data collected during three surveys and with Italian and European data.

Methods

The surveys took place in October 2011, November 2012 and November 2013 in the FUH, a tertiary care hospital with 857 beds in 2011 and, after moving to a new hospital in 2012, with 711 beds. The materials and tools developed for the ECDC PPS of HAI and AM use in acute care hospitals were used for these surveys: the PPS protocol and codebook v4.2, including the case definitions of HAI, PPS data entry forms in an editable format for translation purposes, PPS hospital software HELICSWin.net, User manual – PPS hospital software HELICSWin.net [10]. All acute wards were included, except for Day-surgery and Day-Hospital departments. The study included all patients admitted to the ward before or at 8 a.m. and not discharged from the ward at the time of the survey, including neonates, if born before/at 8 a.m. For each ward, data had to be collected in a single day. Data collection for each survey was completed in two weeks. The surveys were carried out by trained medical doctors of the Postgraduate School of Hygiene and Preventive Medicine of Ferrara University, supported by doctors and nurses of the Hospital Network for Infection Control of each ward. The ECDC standard "Patient data form" was used, structured according to the following sections: demographic data, admission data, clinical data, AM use and HAI data [10].
Demographic, admission and clinical data, useful for identifying patient-based denominator data and risk factors, included: ward name, survey date, patient counter, age, sex, date of admission, surgery since admission, McCabe score [12], invasive devices in place on survey date (central vascular catheter-CVC, peripheral vascular catheter-PVC, urinary catheter, intubation). Only any active HAI on the survey date was recorded on the form [10].
Data collected for HAI included: presence of a relevant invasive device before onset (intubation for PN, central vascular catheter / peripheral vascular catheter for bloodstream infection-BSI and urinary catheter for UTI) [13], HAI present at admission, date of onset, origin of infection (if bloodstream infection, source) and microorganisms data.
AM data (including generic or brand name, route, indication, diagnosis/site of infection, reason) were collected when a patient was receiving an AM on the day of survey (or in the 24 hours before the day of the survey for surgical prophylaxis). Registered drugs were classified according to the Anatomical Therapeutic Chemical (ATC) classification [14]. AMs included in the survey were Anatomical Therapeutic Chemical classes J01 (antibacterials), J02 (antifungals) and J04 (antimycobacterials). Indication for use of systemic AMs was recorded according to the following classification: community-acquired infection, infection acquired in long-term care facility (e.g. nursing home) or chronic-care hospital, acute hospital acquired infection, surgical prophylaxis (single dose, one day, more than one day), medical prophylaxis, other indications, unknown indication/reason, unknown/missing information on indication not verified during survey [10]. Data were collected using the standard ECDC software HELICSWin.net v. 1.3. Statistical analysis was performed using Stata v.13. Difference in the distribution of nominal variables was assessed using Pearson's chi-square test with significance level set at 0.05. Continuous variables were tested for normality of distribution both graphically and by means of Shapiro-Wilkinson test, difference in distribution was then tested using Kruskal-Wallis test. Prevalence rate of HAI was calculated as the percentage of infected patients over the total number of patients observed during each survey. AM use prevalence was calculated as the percentage of the number of patients receiving at least one AM over the total number of patients observed. Risk factors analysis were performed by means of logistic regression in relation to two outcomes: presence of at least one HAI and receipt of at least one AM.
Continuous variables were recoded into categories in order to maintain consistency with ECDC PPS [15] and to address the influence of outliers. The final models for both outcomes were developed by adding those risk factors which resulted to be significant ($P < 0.2$) in univariate analysis in a forward stepwise manner [16]. Significance level for inclusion in final model was set at $p < 0.05$. The presence of a central vascular catheter or peripheral vascular catheter was excluded from both models because of the correlation with the parenteral administration of AMs. Presence of relevant invasive devices was considered before the onset of an HAI for the HAI regression model. Length of stay in the HAI model was considered until the date of HAI onset if an HAI occurred during current hospital stay. Goodness-of-fit was assessed on eight smaller random sub-samples of the data using the Hosmer–Lemeshow chi square test. The discriminatory accuracy of the multiple logistic regression models was assessed using receiver operating characteristic (ROC) analysis. Standardized prevalence rates were calculated by using a 2-step method which takes into consideration predicted probabilities of the outcome according to the regression model and indirect standardization. The predicted probabilities were used to determine the mean predicted risk of HAI or AM use for each survey. Risk index ratios were calculated by dividing the observed (unadjusted) prevalence rates by the mean predicted risk of each survey, and adjusted prevalence rates

were determined by multiplying standardized ratios by the observed prevalence rates in the entire study sample.

Results

Overall, 1,239 patients were observed in the three surveys; the mean age was 62.6 years and 47.3% were male. Mean length of stay was 9.4 days (median 6 days). At the time of survey, a central vascular catheter was present in 20.2% of observed patients; a peripheral vascular catheter in 56.0%; a urinary catheter in 35.9% and the percentage of mechanically ventilated / intubated patients was 3.8%. Differences among data collected during the three surveys proved to be statistically significant ($p < 0.05$) for: presence of peripheral line, presence of central line, McCabe score and surgery since admission. The overall prevalence of HAI was 9.6%, with a total number of 49 HAIs in 2011, 37 in 2012, and 54 in 2013 (HAIs to patients ratio: 1.1 in 2011, 1.1 in 2012, 1.3 in 2013). Case-mix corrected prevalence rates were: 10.1% for 2011, 8.9% for 2012 and 9.6% for 2013. UTIs were the most common HAI in all three surveys, followed by PN (in 2011 and 2012) and bloodstream infections in 2013 (Tab. I). A total of 82.8% HAIs originated in the current hospital. Regression analysis of risk factors associated with the onset of at least one HAI shows statistical significance for: length of stay at risk 4-7 days (OR: 1.9, 95%CI 1.1-3.4; $p = 0.030$), length of stay at risk 8-14 days (OR: 2.3, 95%CI 1.2-4.3; $p = 0.010$) and length of stay at risk > 3 weeks (OR: 3.8, 95%CI 2.1-7.1; $p < 0.001$); McCabe score "Rapidly fatal disease" (OR: 2.4, 95%CI 1.5-3.8; $p < 0.001$); use of urinary catheter (OR: 2.5, 95%CI 1.6-3.7; $p < 0.001$); mechanical ventilation (OR: 2.3, 95%CI 1.1-4.5; $p = 0.023$). The prevalence of HAI was higher in Intensive Care Units in all three surveys.

At the time of the surveys, results for microbiological investigation were available for 120 HAIs (85.0%). *Escherichia coli* was the most common pathogen, followed by *Klebsiella pneumoniae* and *Enterococcus faecalis* (Tab. II). *Escherichia coli* was the most prevalent pathogen even when stratifying by survey and also the most frequent causative pathogen for UTI. During the 3-year study period, isolated strains of *Escherichia coli* were frequently third-generation cephalosporin resistant (range 10%-20%), but only in 2011 were they also carbapenem resistant. In 2011, 33.3% of *Klebsiella pneumoniae* strains were third-generation cephalosporin resistant and 16.7% were carbapenem resistant. Overall, the AM use prevalence was 51.1% (at least one AM). A total of 858 AMs were administered (Tab. III). Parenteral administration was the most prevalent route (69.0% in 2011, 74.0% in 2012 and 79.3% in 2013). AMs were mainly administered for treatment of an infection (relative frequency 61.0% in 2011, 56.2% in 2012 and 70.7% in 2013) and among these mainly for treatment of community acquired infections (57.6% in 2011, in 2012 59.1%, in 2013 60.1%). Surgical prophylaxis was mostly prescribed for more than one day (relative frequency: 65.4% in 2011, 72.0% in 2012 and 88.9% in 2013). Single dose prophylaxis was prescribed in 23.1% in 2011, 20.0% in 2012 and 11.1% in 2013 (relative frequency). One-day surgical prophylaxis was the least frequently prescribed. Prescription for medical prophylaxis was 19.8% in 2011, 24.9% in 2012, 15.0% in 2013. Considering all three surveys, antibacterials for systemic use (ATC group J01) accounted for 93.7% of all prescriptions. AMs most frequently administered were: J01MA fluoroquinolones (21.7% in 2011, 23.0% in 2012, 21.8% in 2013), J01CR combinations of penicillins including beta-lactamase inhibitors (20.4% in 2011, 19.2% in 2012, 21.8% in 2013), J01DD third-generation cephalosporins (22.7% in 2011, 16.6% in 2012, 16.8% in 2013). Fluoroquinolones were the most commonly used AMs in symptomatic lower UTI (total 28.8%) and PN (total 24.5%), including both community acquired infections and HAI. Risk

Tab. I. Characters of Healthcare associated infections (HAIs).

HAI data	Year of survey		
	2011 (N = 450[a])	2012 (N = 379)	2013 (N = 407)
HAI Prevalence (at least one HAI) %	10.0	8.7	10.1
Total number of HAIs	49	37	54
Infection Site - No. (%) of HAI by year of survey:			
Urinary tract infections	18 (36.7)	9 (24.3)	22 (40.7)
Pneumonia	7 (14.3)	9 (24.3)	6 (11.1)
Bloodstream infections (BSI)	5 (10.2)	2 (5.4)	10 (18.5)
Surgical site infections	4 (8.2)	4 (10.8)	3 (5.6)
Gastro-intestinal system infections	5 (10.2)	2 (5.4)	2 (3.7)
Other lower respiratory tract infections	2 (4.1)	1 (2.7)	2 (3.7)
Catheter-related infections w/o BSI		2 (5.4)	
Other	8 (16.3)	8 (21.6)	9 (16.7)

[a] 3 missing records excluded

Tab. II. Top five microorganisms isolated in healthcare-associated infections and percentage of antimicrobial resistance markers.

Microorganisms	No. of isolated microorganisms by year of survey		
	2011 (N = 74)	2012 (N = 28)	2013 (N = 73)
Escherichia coli (%C3G-R) (%Car-R)	24 (16.7) (16.7)	10 (20.0) (0.0)	20 (10.0) (0.0)
Klebsiella pneumoniae (%C3G-R) (%Car-R)	6 (33.3) (16.7)	4 (0.0) (0.0)	6 (0.0) (0.0)
Enterococcus faecalis	2	5	5
Candida albicans	5		6
Staphylococcus epidermidis	1	1	6

C3G-R, Third-generation cephalosporin resistance

Car-R, Carbapenem-resistant

Tab. III. Characters of Antimicrobials (AMs).

AM use data	Year of survey		
	2011 (N = 450[a])	2012 (N = 379)	2013 (N = 407)
AM use prevalence (at least one AM) %	54.4	50.1	48.4
Total number of AM	313	265	280
Top ten antimicrobials agents (ATC codes) - No. (%) of AM by year of survey:			
J01MA Fluoroquinolones	68 (21.7)	61 (23.0)	61 (21.8)
J01CR Combinations of penicillins, incl. beta-lactamase inhibitors	64 (20.4)	51 (19.2)	61 (21.8)
J01DD Third-generation cephalosporins	71 (22.7)	44 (16.6)	47 (16.8)
J01GB Aminoglycosides	13 (4.2)	17 (6.4)	17 (6.1)
A07AA Intestinal anti-infectives antibiotics	7 (2.2)	3 (1.1)	2 (0.7)
J01DB First-generation cephalosporins	23 (7.3)	11 (4.2)	6 (2.1)
J01DH Carbapenems	9 (2.9)	11 (4.2)	20 (7.1)
J01XA Glycopeptide antibacterials	11 (3.5)	16 (6.0)	13 (4.6)
J01XD Imidazole derivatives	7 (2.2)	8 (3.0)	13 (4.6)
J02AC Triazole derivatives	9 (2.9)	10 (3.8)	7 (2.5)
J01FA Macrolides	12 (3.8)	9 (3.4)	4 (1.4)

[a] 3 missing records excluded
ATC, Anatomical Therapeutic Chemical

factors associated with administration of at least one AM showing statistical significance in the regression model were: patient located in surgical ward (OR: 1.7, 95%CI 1.1-2.7; $p = 0.010$) and Intensive Care Unit (OR: 2.7, 95%CI 1.2-6.0; $p = 0.015$); length of stay 4-7 days (OR: 1.4, 95%CI 1.1-1.9; $p = 0.016$); length of stay 8-14 days (OR: 1.6, 95%CI 1.1-2.2; $p = 0.010$); patient underwent non-NHSN/minimal surgery during current hospitalization (OR: 1.5, 95%CI 1.1-2.2; $p = 0.013$); use of urinary catheter at the time of survey (OR: 1.9, 95%CI 1.4-2.4; $p < 0.001$); mechanical ventilation at the time of survey (OR: 2.6, 95%CI 1.1-6.0; $p = 0.030$). Case-mix corrected AM use prevalence rates were: 54.2% in 2011, 50.5% in 2012 and 47.9% in 2013.

Discussion

The described prevalence rate of nosocomial infections was higher than the values reported in other studies [17-21] including the ECDC's 2011 report [15], which estimates a prevalence rate of 6.0% (country range 2.3%–10.8%) in European acute-care hospitals (6.1% in Italy). This difference in the reported values is due in part to the different characteristics of the hospitals included in the European survey which collects results from primary, secondary, tertiary care and specialized hospitals in different countries. However, the prevalence rate of HAI in FUH remains higher even when comparing results from tertiary care hospitals only (7.2%). One possible reason may be the fact that the surveys were carried out by independent auditors, to avoid conflicts of interest and to ensure the integrity of the auditing process. As con-

firmed by existing literature, Intensive Care Units were the most affected wards [15, 17-21]. UTIs were the most common HAI in all three surveys in FUH, unlike what is reported in other studies where PN and surgical site infections were more prevalent [15, 17, 18]. Use of urinary catheter, a well known risk factor for UTIs [22-24], was higher than what is reported in the literature [15, 19, 21]. Prevalence of surgical site infections was found to be lower than what is reported by other similar surveys [15, 17-21]. Appropriate urinary catheter indication is certainly an area which requires further analysis to assess possible overuse and guide practical interventions [25]. Year by year comparison of nosocomial infections and risk factors in the three surveys delivers substantially constant results even when corrected for case-mix by means of logistic regression. Risk factor analysis is consistent with data in the literature [15, 19, 21]. Statistically significant risk for HAI occurrence is independently associated with increased length of stay, McCabe Score "Rapidly fatal disease", use of urinary catheter and mechanical ventilation. Mechanical ventilation associated risk suggests a need for more effective preventive measures against ventilator-associated infections [26]. At the time of the surveys, results for microbiological investigation were available for 120 HAIs (85.0%). *Escherichia coli* was the most frequent microorganism isolated in all three surveys and the most frequent causative pathogen for UTI, followed by *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Candida albicans*. These results show a higher prevalence of *Enterobacteriaceae* when compared with the ECDC's report data [15] which can be explained by the higher frequency of UTIs in FUH. AM use rates were higher than those reported in the literature [15, 19], while the average number of AMs to treated patients ratio is consistent with the value reported by ECDC [15], showing no evidence of a higher rate of multidrug protocol prescriptions in FUH. Fluoroquinolones, third-generation cephalosporins and combinations of penicillins (including beta-lactam inhibitors) were the most frequent AM prescribed in all three surveys, a similar result to other literature reports which further underline a widespread use of broad spectrum antibiotics combined in multidrug protocols that is often necessary to counteract the increasing prevalence of AM resistance [15, 17-19, 27]. On the other hand, the excessive and inappropriate use of antibiotics is the prime mover of the rapidly increasing prevalence of antibiotic-resistant microorganisms [28, 29]. AMs were mainly prescribed to treat an infection (mainly community acquired). Medical prophylaxis was the second most frequent indication in all three surveys. These results are similar to those reported by the ECDC's 2011 point prevalence survey for Italy [15]. Surgical prophylaxis was mostly prescribed for more than one day, while one-day surgical prophylaxis was the least frequently prescribed. These results are substantially similar to those reported by ECDC for Italy in 2011 and other similar studies [15, 18, 19], underlining that antibiotics are used for longer than what is suggested by the international consensus [30], further stressing the need for specific stewardship programs [31, 32]. Year by year analysis shows a decreasing, although not statistically significant, prevalence of AM prescription in FUH, dropping from 54.4% in 2011 to 48.4% in 2013, a result confirmed by standardization through logistic regression model. AM stewardship is a critical area of intervention in FUH, aimed at changing prescribing practices, leading to a better control of drug resistant microorganisms, improved appropriateness of antibiotic use and decreased costs.

Conclusions

FUH has a long history of activities aimed at risk management and infection control, based on a multimodal and multidimensional approach [11]. Moreover, the hospital's infection control policy includes: audit and feed-back to improve compliance of the healthcare workforce to good practices; retraining courses and educational programs; drafting reminders to support good practices for workers, patients and caregivers; continuous surveillance of surgical site infections; active support for the WHO Campaign "Save lives: clean your hands" since 2006, with the participation as an international site in the experimentation of WHO Guidelines on Hand Hygiene in Health Care (Advanced Draft) [33, 34]. Despite their limitations, PPS are not expensive, take little time to carry out and need few human resources. PPS are easy repeatable and provide meaningful information to use for specific targeted interventions. The yearly repetition will be a useful means of keeping interest alive on the subject of HAI and AM use [35] and highlighting how changes in healthcare practices affect outcome variables.

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Authors' contributions

PA, GG, AS, MCM was responsable for the research coordination and contributed to the protocol definition, data collection, data analysis, manuscript drafting and critical revision of the manuscript. BB, AV, AF contributed to the data collection, data analysis and critical revision of the manuscript. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Operating room environment and surgical site infections in arthroplasty procedures

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Keywords

Operating room • surgical site infection • surveillance • arthroplasty procedures

Summary

Background. *The rate of surgical site infections (SSI) is strongly influenced by operating room quality, which is determined by the structural features of the facility and its systems and by the management and behavior of healthcare workers. The aim of the present study was to assess microbial contamination in the operating room during hip- and knee-replacement procedures, the behavior of operating room staff and the incidence of SSI through post-discharge surveillance.*
Methods. *Microbial contamination was evaluated by active and passive sampling at rest and in operating conditions. Organizational and behavioral characteristics were collected through observational assessment. The incidence of SSI was evaluated in 255 patients, and follow-up examinations were carried out 30 and 365 days after the procedure.*
Results. *The mean values of the airborne and sedimenting microbial loads were 12.90 CFU/m^3 and 0.02 CFU/cm^2/h, respectively. With regard to outcome, the infection rate proved to be 0.89% and was associated with knee-replacement procedures. The microorganism responsible for this superficial infection was Staphylococcus aureus.*
Conclusions. *Clinical outcomes proved to be satisfactory, owing to the limited microbial load (in both at-rest and operating conditions), the appropriate behavior of the staff, compliance with the guidelines on preoperative antibiotic prophylaxis, and efficient management of the ventilation system.*

Introduction

The rate of surgical wound infections is strongly influenced by operating room quality, which is determined by the structural features of the facility and its systems and by the management and behavior of healthcare workers [1, 2]. It has been suggested that the main sources of contamination, especially in clean surgical procedures, are the patient's skin and airborne particles from operating room personnel [2, 3]. In this regard, a study conducted by the Medical Research Council showed a correlation between microbial air contamination and the incidence of surgical site infections (SSI) in prosthetic joint surgery [4]. Hip- and knee-replacement operations are common procedures and are performed to improve quality of life in individuals with end-stage joint degeneration. However, SSI can give rise to very severe complications which nullify the efficacy of the procedure. Infection rates after primary total knee arthroplasty reported in the literature range from 0.39% to 2.5%; total hip infection rates are approximately 0.2%-2.2% for primary procedures [5]. In addition to the devastating consequences for the patient, such infections have an enormous economic impact on the treating hospital, since they substantially prolong hospitalization and increase costs [6]. Approximately 12,000 joint infections occur annually in the United States, with an estimated cost of $600 million a year [5].

A number of host factors increase the risk of treatment failure, including male sex, advanced age, rheumatoid arthritis, an American Society of Anesthesiologists (ASA) risk score > 2, diabetes mellitus, morbid obesity, immuno-compromission and previous revision arthroplasty [7, 8]. Other factors related to the risk of infection concern the pathogen involved, medical therapy and surgical techniques [9-11]. The aim of the present study was to assess microbial contamination in the operating room during hip- and knee-replacement procedures, the behavior of staff and the incidence of SSI through post-discharge surveillance.

Materials and methods

The study started on 1st October 2014 and was concluded on 31st January 2016. The study evaluated microbial contamination in the operating room during 255 operations (hip- and knee-replacement surgery; ICD9-CM 81.51 and 81.54), and microbial contamination in at-rest conditions at the beginning of each operating session.
The operating room is devoted exclusively to prosthetic surgery and situated within a hospital facility in the north-west of Italy.
The incidence of SSI was evaluated in the patients, and follow-up examinations were carried out 30 and 365

days after the procedure. For each of the 255 procedures monitored, the following patient characteristics were recorded: age, sex, ASA score, type of prosthesis implanted and antibiotic therapy. With regard to the surgical teams (n = 2) involved in the procedures, several behavioral features were monitored.

Features of the operating room and the ventilation system

The design of the operating suite provides adequate space for reception, anesthesia, surgery, recovery, and observation of patients. The operating room has a turbulent-flow ventilation system equipped with High Efficiency Particulate Air filter (HEPA) filters, which are 99.97% efficient in removing airborne particles of 0.3 μm or larger; the filters are replaced every 6 months and maintenance work on the system is carried out periodically in accordance with a predetermined schedule.
The operating room is under positive pressure in relation to the adjacent rooms (≥ 5 Pa).

Environmental features

Airborne bacterial contamination in the center of the room in operating conditions

To determine the total airborne bacterial load, we used an SAS SUPER 100 (PBI International®) impactor equipped with RODAC plates (Ø = 55 mm). In order to sample the air in the center of the room, the instrument was positioned in the immediate vicinity of the operating table, at a height of 1.5 m. During each procedure, a 1000 L volume of air was aspirated by means of a multi-aspiration modality; the impactor was switched on by remote control just as the skin was incised, and was switched off on completion of suturing. In addition, passive air sampling was carried out during each procedure. Settle plates (9 cm in diameter) were left open to the air according to the 1/1/1 scheme (for 1 h, 1 m from the floor, about 1 m from any obstacles) to determine the index of microbial air contamination (IMA).

Airborne bacterial contamination in at-rest conditions

In order to assess the efficacy of the ventilation system used in the operating room, contamination of the air emerging from the inlet ports was evaluated by means of an SAS SUPER 100 (PBI International®) impactor equipped with RODAC plates (Ø = 55 mm) before the beginning of each session of operations. A total volume of 1000 L of air was aspirated at each inlet port.
In order to sample the air in the center of the room in at-rest conditions, we used an SAS SUPER 100 (PBI International®) impactor equipped with RODAC plates (Ø = 55 mm). The instrument was positioned in the center of the operating room, at a height of 1.5 m. A total volume of 1000 L of air was aspirated.
To measure the total airborne bacterial count, γ-irradiated tryptic soy agar (TSA) (Biotest Italia s.r.l.) was used. Plates were incubated at 37°C for 48 h before the total bacterial count was measured [12]. Microbiological results were expressed as CFU (colony forming units)/m^3 and CFU/m^2/h for active samplers and settle plates, respectively.

Surface bacterial contamination

Microbial measurements of surfaces were conducted with RODAC contact plates (Ø = 55 mm) containing Columbia blood agar culture medium (Biotest Italia s.r.l.). Sampling was carried out after sanitization of the operating room as indicated by ISPESL and by the French guidelines [13, 14].
Plates were incubated at 37°C for 48 h before the total aerobic bacterial count was measured. Microbiological results are expressed as CFU (Colony Forming Units)/plates.

Microclimatic parameters

With regard to the detection of microclimatic parameters (temperature; relative humidity; air speed) we used a portable microclimatic BABUC (LSI©) device equipped with psychrometric probes, a black-globe thermometer and a hot-wire anemometer; the device was positioned in the vicinity of the operating table. A sufficient time was allowed for the probes to acclimatize; the instrument then recorded microclimatic parameters for the entire duration of the surgical activity.
The comfort indexes Predicted Mean Vote (PMV) and Predicted Percentage of Dissatisfied (PPD) were calculated by means of Bruel & Kjær software by entering the data of M (metabolism), Icl (clothing), ETA (mechanical efficiency) relative to the surgical staff and environmental parameters (temperature, relative humidity, air speed, etc.).

Number of efficacious air exchanges

The efficacy of the air-conditioning system was assessed in at-rest conditions by measuring the decay of the concentration of tracer gas by means of a portable GA301 meter (Eco-CONTROL, Milan) connected to a computer for the collection and analysis of data, as described by Sartini et al. [15].

Organizational and behavioral characteristics

During each operation, we collected detailed information on the surgical procedure, including the duration of the procedure (skin-skin), the number of staff members in the room at the time of the incision, and the door-opening rate. For each surgical team, we also recorded the adherence to dress regulations and preoperative antibiotic prophylaxis protocol, behavioral aspects, etc.

Follow-up

In order to detect any surgical site infections, surveillance examinations were carried out 30 and 365 days post-operatively. The extended period of ascertainment of nosocomial SSI of up to 1 year was set in accordance with the Centers for Disease Control and Prevention (CDC) for operative procedures such as replacement of the hip and the knee by artificial joint prostheses [16].
The first control (day 30) involved an outpatient examination; subsequently, telephone interviews were conducted

by trained healthcare personnel, who utilized a standard data-collection form that had already been validated in previous studies on SSI [17]. Patients had been informed of the postoperative epidemiological surveillance that they were to undergo 365 days post-operatively.
SSI detection was carried out in accordance with the definition laid down by the National Nosocomial Infections Surveillance (NNIS), which has also been adopted by Hospitals in Europe Link for Infection Control through Surveillance (HELICS) [18]. SSI are defined as infections occurring within 30 days after a surgical operation (or within one year if an implant is left in place after the procedure) and affecting either the incision or deep tissue at the operation site [19].

Statistical methods

Statistical analysis was carried out by means of the STATA SE14™ software (StataCorp LP - USA). As the data did not display a normal distribution, every possible numerical transformation of the data was evaluated. As none of these was able to reduce the effect of skewness, the data were analyzed by means of non-parametric tests. The results were analyzed in terms of descriptive statistics, and the relationships between data were examined by means of the non-parametric Mann-Whitney-Wilcoxon ranksum test and Pearson's Chi-square test.

Ethics Statement

As the study was carried out as part of routine control tests that we conduct in the operating rooms of the hospital, no ethics approval was needed. As is the case of all studies conducted in the hospital environment, the General Management of the hospital approved the study protocol. The General Management is responsible for ensuring the ethical aspects of all activities of the hospital. Furthermore, the entire study was organized in accordance with a protocol agreed upon with the operating room teams. On entering the hospital, all patients sign an informed consent form regarding treatments in the hospital and the conditions of those treatments. Finally, the research was carried out in full respect of the Italian law on the privacy (Legislative Decree N. 196 of 30th June 2003).

Results

Of the 255 procedures monitored, 49.0% involved total hip replacement (ICD9-CM:81.51) and 51.0% total knee replacement (ICD9-CM:81.54). Regarding the duration of total hip replacement and total knee replacement procedures, the median values were 35 (range 17-126) and 39 minutes (range 19-102), respectively; the difference between these values did not prove statistically significant ($z = -1.28$, $p = 0.20$). Concerning the characteristics of the prostheses implanted, 80% were metal-polyethylene, 7.5% metal-metal, 5.1% ceramic-ceramic, 3.5% metal-ceramic, 3.1% ceramic-polyethylene, 0.39% ceramic-metal and 0.39% Titanium. In 38.4% of cases, the prosthesis was fixed by means of cement, and in 86.7% tobramycin was added. For what concerns the environmental features of the operating room, the values of the airborne and sedimenting bacterial loads and microclimate parameters are reported in Table I.

Tab. I. Mean values, standard deviation and range of airborne and sedimenting bacterial load (during procedures), of microclimate parameters and microclimate indexes in the operating room.

	Procedures	Mean±SD	Min-Max
Airborne bacterial load, center of room (CFU/m³)	All procedures	12.90±17.00	0-85
	Total hip replacement	12.18±12.97	0-80
	Total knee replacement	13.58±20.16	0-85
Sedimenting bacterial load (CFU/cm²/h)	All procedures	0.02±0.03	0-0.13
	Total hip replacement	0.02±0.03	0-0.13
	Total knee replacement	0.02±0.02	0-0.09
Microclimate Environmental parameters	All procedures	18.94±1.16* 50.65±14.37^ 0.06±0.02°	16.38-20.45* 21.3-73.6^ 0.03-0.11°
	Total hip replacement	18.48±1.60* 45.72±17.93^ 0.06±0.01°	16.38-20.11* 21.3-62.2^ 0.05-0.07°
	Total knee replacement	19.24±0.81* 53.93±12.11^ 0.07±0.03°	18.19-20.45* 36.4-73.6^ 0.03-0.11°
Microclimate Indexes	All procedures	0.21±0.13** 6.3±1.5°°	0.03-0.44** 5-9°°
	Total hip replacement	0.20±0.08** 5.7±0.5°°	0.07-0.25** 5-6°°
	Total knee replacement	0.22±0.17** 6.7±1.9°°	0.03-0.44** 5-9°°

*Air temperature (°C); ^relative humidity (%); °air speed (m/s); **PMV surgical staff, °°PPD surgical staff (%)

As can be seen, the highest mean values of the airborne bacterial load (13.6 ± 20.2 CFU/m^3) were recorded during total knee replacement procedures, while the mean values recorded during total hip replacement proved to be lower (12.2 ± 13.0 CFU/m^3). In 63.01% of total hip replacement procedures, mean values of airborne bacterial load below 10 CFU/m^3 were recorded; in total knee replacement procedures, the corresponding percentage was 73.39%.

The mean values of the sedimenting bacterial load did not differ between the two types of procedure. The surface bacterial load was always 0 CFU/plate.

With regard to microclimatic parameters, considering the total number of procedures, the mean values of air temperature, relative humidity and air velocity were: 18.94 ± 1.16°C; 50.65 ± 14.37% and 0.06 ± 0.02 m/s, respectively. No statistically significant difference emerged between the two types of procedures ($p > 0.05$).

With regard to the characteristics of the air-conditioning system, 19 efficacious air exchanges were carried out per hour.

On visual inspection carried out at the beginning of each surgical session, the overhead light and the grills of the inlet ports of the air-conditioning system were free from visible dust. For all operating sessions the microbial load of the airflow through the inlet ports and in the air (at-rest conditions) proved to be <1 CFU/m^3 and 4 ± 2 CFU/m^3, respectively.

Concerning the organizational and behavioral features of the staff during the procedures, surgeons wore highly effective isolation helmet systems and the instrument-keeper wore headwear and a semi-integral mask; anesthetists and circulating nurses wore surgical masks and hair covering. The surgical technique utilized in all the procedures monitored involved the use of the ultrasonic scalpel.

The doors communicating with the rooms adjacent to the operating room were kept closed; the door-opening rate was 0.24 times per minute. The mean number of persons present in the operating room was 5 ± 1.

With regard to patient characteristics, 39.61% were males and 60.39% females; the mean age of the overall patient population was 68.55 ± 10.61 years (range 42-91): 70.79 ± 8.27 for women and 65.14 ± 12.71 for men. ASA scores were: 1 in 10.20% of patients, 2 in 62.35% and 3 in 27.45%. The difference between the distribution of ASA scores in the two types of procedure (hip and knee replacement) did not prove statistically significant ($X^2 = 2.2530$, $p = 0.336$).

All of the patients examined had received preoperative antibiotic therapy 30-60 minutes prior to skin incision. Table 2 shows the drugs used and their doses. A further dose of antibiotic was administered to 48.84% of patients within 24 hours after surgery.

With regard to follow-up, 255 patients were examined in the hospital 30 days after the procedure. After 365 days, 84.71% responded to follow-up. Within the first 30 days of follow-up, 3.53% of patients had taken additional antibiotic therapy for 1 week. However, this was for reasons unconnected with the procedure (infections of the respiratory and/or urinary tracts). Only one patient, who had undergone a knee-replacement procedure, presented with a superficial *S. aureus* infection of the wound; this resolved rapidly.

Tab. II. Percentage use of antibiotics for preoperative prophylaxis.

Antibiotic used	%
Vancomycin 1 g associated to Pefloxacin 400 mg	96.08
Cefazolin 2 g associated to Amikacin 500 mg	3.14
Cefazolin 2 g associated to Pefloxacin 400 mg	0.78

Discussion and conclusions

An incidence of surgical site infections of 0.3-2.5% after arthroplasty procedures of knee and hip has been reported [20]. In our study, only one case of superficial infection was recorded; this was in a patient who had undergone knee-replacement surgery. The infection rate in knee-replacement procedures therefore proved to be 0.89% when calculated on the number of responders at 365 days. The microorganism responsible for this superficial infection was *S. aureus*, one of the most common infecting organisms after periprosthetic joint surgery [18, 21]. This infection rate is in line with that reported in the literature [6, 22].

No postoperative infections were recorded in the sample of responders who had undergone hip-replacement procedures.

The clinical outcome recorded may have been influenced by a number of factors, including the microbiological characteristics of the operating room.

In the present study, the mean values of the airborne microbial load (12.90 ± 17.00 CFU/ m^3) during all procedures proved to be below the standard values (180 CFU/m^3) for conventionally-ventilated operating rooms in Italy [13]. Moreover, during most replacement procedures, the airborne microbial load was below the limit of 10 CFU/m^3 indicated by United Kingdom's National Health Service (NHS) for ultra-clean operating rooms with unidirectional airflows, which is recommended for arthroplasty procedures [23].

In this regard, it has been shown [24] that there is a progressive fall in the incidence of joint sepsis, especially when air contamination is below 10 CFU/m^3. The mean value of the sedimenting bacterial load was 0.02 CFU/cm^2/h, corresponding to 1 IMA/h; this is below the 2 IMA/h threshold indicated by the Association of Swiss Hospitals for operating rooms in which orthopedic prosthetic surgery is performed [25].

The good levels of airborne microbial contamination were achieved despite the fact that the ventilation system provided turbulent, not laminar, airflow. This can probably be attributed to several factors.

The fact that the technical department carefully scheduled cleaning operations (both of the conduits of the ventilation system and of the grills of the inlet ports) and the replacement of filters may have played an important role in abating the microbial load of the air supplied. The use of a laminar-flow system should improve the microbiological quality of the air, thereby further reducing the risk of SSI in prosthetic orthopedic surgery.

The results regarding surface bacterial contamination highlight the fact that the efficacy of sanitation procedures reduces the risk of cross-infections [26].

The clinical outcomes reported could have been partially affected by the thermal comfort of the surgical staff, as emerged from PMV and PPD values, which were within the reference values indicated by Fanger; indeed, thermal comfort improves concentration, reducing mistakes and accidents [27].

Providing proper ventilation is only one aspect of a complex strategy to minimize the risk of infection during surgical operations [28-30]; procedural and behavioral factors can also have a negative impact on the surgical outcome, including the risk of SSI.

A behavioral approach aims to reduce the number of airborne particles in the operating room through disciplinary measures. Some authors have observed that simple and cheap measures, such as limiting the number of staff members in the operating room and restricting their movements to a minimum, can reduce the dispersion of microbes in the air [31]. During the present study, operating room staff kept their movements to a minimum and were always properly attired.

Knobben et al. [32] observed that the combination of systemic and behavioral measures in the operating room, such as wearing proper attire and limiting needless activity, led to a reduction in the incidence of intra-operative bacterial contamination and, consequently, of prolonged wound discharge and superficial SSI. Moreover, after one-year follow-up, fewer deep periprosthetic infections were recorded. While it is difficult to determine the relative influence of each individual measure on the final result, the combination of all these parameters evidently creates the most effective weapon against infections.

We cannot rule out the possibility that appropriate behavior on the part of surgical teams during the study might have been influenced by the so-called "Hawthorne effect", i.e. the notion that performance improves when subjects are aware that they are being observed.

The clinical outcomes achieved seem to be explained by the microbial load (in both at-rest and operating conditions), the appropriate behavior of the staff, compliance with the guidelines on preoperative antibiotic prophylaxis, and efficient management of the ventilation system.

In this regard many studies have shown that various methods can be adopted in order to minimize postoperative infection [20, 33, 34]; these include using antibiotic-impregnated cement and laminar air flow, and minimizing operating room traffic. However, one of the most effective ways to prevent infection has proved to be the administration of prophylactic antibiotics within 1 hour of surgical incision and continuation of its use during the immediate postoperative period [35, 36]. The importance of timing the first dose correctly is now underlined in the official recommendations for good clinical practice, so much so that in the United States this concept has been incorporated into "pay-for-performance" measures [37].

It is currently estimated that antibiotic prophylaxis in prosthetic surgery is able to prevent one infection for every 13 patients to whom it is administered [38]. The unequivocal evidence of the efficacy of perioperative antibiotic prophylaxis has led to this practice being adopted as standard treatment in these categories of patients, and great efforts have been made to raise awareness of this issue among all the health-care workers involved, with a view to ensuring efficacious administration [39].

Various international bodies [17, 33, 40, 41] recommend the use of glycopeptides for prophylaxis in high-risk procedures involving the implantation of prosthetic material whenever SSI due to MRSA are seen to be particularly frequent. In the hospital facility that we monitored, the decision to use Vancomycin in such a high percentage of cases was driven by the epidemiological assessment of the spread of MRSA in the hospital and/or by the risk factors for MRSA colonization in these patients.

The surveillance of postoperative infections is an essential tool in the management of infective risk. Published data suggest that as many as 20% to 70% of SSI are detected during the post-discharge period, although post-discharge SSI data are reportedly difficult for many medical centers to collect comprehensively [42].

The department of orthopedic surgery where the present study was carried out is a center of excellence for hip- and knee-replacement surgery; as such, it also receives patients from outside the region in which it is situated. In such cases, the post-discharge course (apart from the outpatient examination 30 days after the procedure) and rehabilitation are often monitored by facilities situated close to the patient's place of residence. It is therefore difficult, especially for hospital facilities with such a large catchment area, to keep track of any postoperative infections that may arise. Consequently, there is a risk of underestimating the real rate of surgical site infections. It was this consideration that prompted us to institute a system of post-discharge surveillance which would, at least in part, fill this gap. The good response obtained from patients through telephone interviews, even a year after the surgical procedure (84.71% of responders), can be ascribed to the fact that, before surgery, patients were carefully informed of the importance of complying with follow-up, the time schedule of telephone contacts, the nature of the questions that would be asked and the purpose behind them.

Thus, the surveillance of SSI requires a systematic approach, with attention to multiple risk factors related to the patient, the procedures, including proper antibiotic prophylaxis, and the hospital environment [43]. While it is difficult to determine the relative influence of each individual measure on the final result, the combination of all these parameters evidently creates the most effective weapon against infections.

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The authors declare no conflict of interest.

Authors' contributions

MLC conceived, designed and coordinated the research. GO and ES collected data and performed the data quality control. MS and ES optimized the informatics database. MS performed the statistical analyses. AMS evaluated the results. MLC and AMS wrote the manuscript. All Authors revised the manuscript and contributed to improving the paper. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Epidemiology and biomolecular characterization of carbapenem-resistant *klebsiella pneumoniae* in an Italian hospital

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Keywords

Carbapenem-resistant Klebsiella pneumoniae • Bloodstream infection • Mortality

Summary

Objective. *To describe the occurrence of CRKP infections in a tertiary care hospital and to analyse the allelic profiles of the clinical strains involved and the most frequent carbapenemases.*
Design. *The study analyzed cases of infection due to CRKP in the period 2013-2014; 147 cases were recorded, most of which (82.31%) were in-hospital infections.*
Setting. *A hospital in northern Italy.*
Methods. *We retrospectively collected: data on patient characteristics and the microbiological characteristics of CRKP. Isolates from 72 of the in-hospital cases underwent molecular typing (MLST); in addition, in each isolate, a procedure for the detection of the blaKPC gene was carried out.*
Results. *The in-hospital death rate was 24.0% in 2013 and 37.5% in 2014. However, the difference between these two values did not prove statistically significant (P > .05).*
Analysis of mortality revealed that bloodstream infections were more frequently associated with death than other infections (χ^2 = 14.57, P < .001). The age-adjusted Cox proportional hazard model revealed that the patients with bacteremia due to CRKP had a 3-fold higher risk of death (HR 3.11; 95% CI 1.66 - 5.84, P< .001) than those with infections of other sites.
MLST revealed that the prevalent allelic profile was ST 512 (79.62%); the most frequent carbapenemase was KPC-3 (83.8%).
Conclusions. *Our results are in line with those of recent studies, which have shown that the spread of CRKP in Italy is a matter of concern and that further efforts have to be made to prevent the potential dissemination of carbapenemase-producing clones of K. pneumoniae, whenever possible.*

Introduction

Since the 1970s, the selective pressure exerted by antibiotics has given rise to bacterial species that are increasingly resistant, and the last 20 years have seen a dramatic rise in the number of multi-resistant pathogenic strains [1]. Multidrug-resistant *Klebsiella pneumoniae* is one of the leading causes of nosocomial infection worldwide. It causes urinary tract infections (UTIs), pneumonia and intra-abdominal infections in hospitalized immunocompromised patients with severe underlying diseases [2] and is responsible for roughly 15% of Gram-negative infections in hospital intensive care units (ICUs) [3].
After the spread of strains resistant to beta-lactams at the end of the 20th century, the diffusion of isolates of *K. pneumoniae* resistant to carbapenems and colistin is now reducing treatment options and the containment of infections [4]. In recent years, carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has become a widespread concern and carbapenemase production mediated by bla_{KPC} is the most prevalent mechanism conferring resistance to carbapenems [5]. Outbreaks of CRKP have increasingly been reported in various healthcare settings [4, 6, 7], including long-term acute care hospitals [8].
Risk factors for colonization and infection with CRKP are similar to those associated with other multidrug-resistant organisms [9]. Lengthy hospitalization, antibiotic use, invasive procedures and admission to the ICU [8] are associated with an increased risk of acquisition of CRKP.
Mortality rates due to infections caused by CRKP are high, ranging from 26% to 44% and reaching 70% in cases of bacteremia [10-12]. However, deaths reported to be associated with carbapenem-resistant *K. pneumoniae* have included several cases in which the patient had a severe underlying disease, and it is frequently difficult to determine whether carbapenem-resistant *K. pneumoniae* infection was the cause of death.
The aim of the present study was to describe the occurrence of CRKP infections in a northern Italian hospital and to analyse the allelic profiles of the clinical strains involved and the most frequent carbapenemases.

Materials and methods

Setting

The study was conducted in a nationally renowned, highly specialized Northern Italian hospital organized

in accordance with treatment intensity. Structured in pavilions, the hospital has 458 beds (mainly located in 3- and 4-bed rooms) and each year carries out over 15,000 ordinary hospitalizations and more than 8600 medical procedures in Day Hospital and Day Surgery settings.

Study design

The study retrospectively analyzed cases of infection due to CRKP in the period 2013-2014.
Patients who were identified as having CRKP infections within the first 72 h of admission were defined as community-associated cases or, if they had been exposed to healthcare settings during the previous three months, imported healthcare-associated cases. Clinical episodes of infection were considered to be hospital-acquired if they were not present at the time of hospital admission and appeared 72 hours after admission.
A record was made of each case patient's age, gender, history of hospitalizations, antibiotic treatments, duration of hospitalization, the date of the first CRKP detection, the site of infection and co-infections, invasive procedures and outcomes.
If a positive patient had been transferred from one ward to another, acquisition of the infection was attributed to the ward in which the diagnosis of infection was made, as the exact site of acquisition could not be determined.
The incidence of infections was calculated per 1000 days of hospitalization.

Classification of patients according to infection risk and type of isolation applied

Patients were grouped into two categories of infection risk: high-risk and medium-risk according to the characteristics of the patient and the site of infection. Patients were deemed to be at high risk if they presented one or more of the following characteristics: presence of excretions/secretions at the infection site, confinement to bed, lack of self-sufficiency, and great need for assistance. Patients in this category underwent structural or cohort isolation; if this was not possible, functional isolation was implemented.
Patients were defined as being at medium risk if they presented one or more of the following characteristics: presence of reduced secretions or excretions at the infection site, capability of temporal and spatial orientation, ability to cooperate, self-sufficiency, and low-medium need for assistance. These patients underwent functional isolation.

Microbiologic methods

Bacterial identification and antimicrobial susceptibility testing were carried out by means of the Phoenix 100 Automated Microbiology System (Becton Dickinson Diagnostic Systems, USA).
Confirmatory MIC testing for imipenem, meropenem and ertapenem was carried out by means of Etest (bioMérieux SA, France) and the Kirby Bauer disk diffusion method [13]. All collected isolates that were confirmed to be non-susceptible to imipenem and/or meropenem and/or ertapenem according to the EUCAST breakpoints [14] were considered to be Carbapenem-resistant *K. pneumoniae* and underwent a modified Hodge test [15] to confirm carbapenemase production. In addition, in order to identify which carbapemenase was present, PCR for the bla_{KPC} gene was carried out [16].

Biomolecular analysis

The isolates from 72 of the in-hospital cases underwent molecular typing by means of the MLST technique (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). This technique involves amplifying and sequencing seven housekeeping genes (rpoB, gapA, mdh, pgi, phoE, infB, tonB) and, through comparison with the data available in an online databank, enables a specific ST (Sequence Type) to be assigned to each isolate. The amplification protocol prescribes an initial denaturing phase at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes.
In addition, in each isolate, a procedure for the detection of the bla_{KPC} gene was carried out through the amplification and sequencing of a DNA fragment of about 1000bp by means of the following primer pairs: bla_{KPC}-Forward 5'-TGTCACTGTATCGCCGTC-3' and bla_{KPC}-Reverse 5'-CTCAGTGCTCTACAGAAAACC-3'. The amplification phase consisted of an initial denaturing phase at 95°C for 5 minutes, followed by 35 cycles at 95°C for 60 seconds, 55°C for 40 seconds and 72°C for 90 seconds, with a final extension at 72°C for 10 minutes. The sequences obtained were compared with those available in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which enabled the bla_{KPC} gene to be characterized.
For both the identification of the Sequence Type and detection of the bla_{KPC} gene, sequencing was carried out by means of an Applied Biosystems 3730xl DNA Analyzer (Life Technologies, USA).

Infection control measures

In all high-risk patients, rectal swabs were taken by the ward nurse on admission to the ward. Cultures were then sent directly to the bacteriology laboratory for prompt CRKP identification.
The diffusion of the microorganism was monitored by means of continuous integrated microbiological surveillance, starting with laboratory data (alert organism surveillance). Following laboratory identification of an epidemiologically important microorganism, the dedicated software of the surveillance system automatically e-mails the data to all the members of the Hospital Infections Committee, who then implement the interventions deemed necessary, with particular regard to the application of isolation measures.
A validated report is simultaneously sent through the laboratory information system to the hospital facility involved.
Antibiotic therapy was instituted after consultation with the infectious-disease specialist on the basis of case history, patients' clinical features, microbiological isolates and antibiotic sensitivities.

Hospital-wide policies to prevent nosocomial carbapenem-resistant Enterobacteriaceae transmissions were introduced on the basis of an institutional protocol that was developed in accordance with the American Centers for Disease Control and Prevention (CDC) indications [17].

Statistical analysis

Statistical analysis was carried out by means of STATA SE13TM software (Stata Corp LP, USA). The results were analyzed in terms of descriptive statistics, and differences between groups were evaluated by means of non-parametric chi-square test and Fisher's mid-P exact test.

Kaplan-Meier survival curves were assessed by means of a log-rank analysis, to compare overall survival, and a COX proportional hazard model, to assess the role of possible confounders. A p-value < 5% was considered significant.

Results

In the period of observation, 147 cases of CRKP infection were recorded (75 in 2013 and 72 in 2014) most of which (82.31%) were in-hospital infections.
The overall mean age of the patients was 78.95 ± 12.05 years (range 26-97 years). Women accounted for 50.34% of the patients; their mean age was 81.49 ± 10.51 years (range 42-97 years). The mean age of the male patients

Tab. I. Carbapenem-resistant *Klebsiella pneumoniae* case characteristics.

	Value	(%)
Total no. confirmed	147	
Unit in which the diagnosis of infection was made		
Medical ward	55	(37.41)
Geriatric unit	53	(36.06)
Surgical ward	30	(20.41)
ICU*	9	(6.12)
Cases from:		
Hospital	121	(82.31)
Other healthcare facility	13	(8.84)
Community	13	(8.84)
Invasive procedures correlated with infection	56	(46.28)
Bladder catheter	42	(75.00)
CVC§	5	(8.92)
ERCP^	3	(5.36)
Tracheal intubation	2	(3.57)
Percutaneous surgical drainage	2	(3.57)
Urethral catheter	1	(1.79)
PIVC°	1	(1.79)
Sites of CRKP infection		
Urinary tract infection	96	(65.31)
Bloodstream infection	23	(15.64)
Surgical site infection	15	(10.20)
Airways	7	(4.76)
Other	6	(4.09)
Specimen type		
Urine from catheter	65	(44.22)
Urine	31	(21.09)
Blood	25	(17.00)
Other	6	(4.08)
Bronchial aspirate	4	(2.72)
Surgical fragment	4	(2.72)
Abdominal fluid	4	(2.72)
Pus	4	(2.72)
Skin swab	1	(0.68)
Wound swab	1	(0.68)
Bile	1	(0.68)
Expectorate	1	(0.68)

*ICU = intensive Care Unit - §CVC = central venous catheter - ^ERCP = Endoscopic Retrograde Cholangiopancreatography - °PIVC = Peripheral Intravenous Catheter

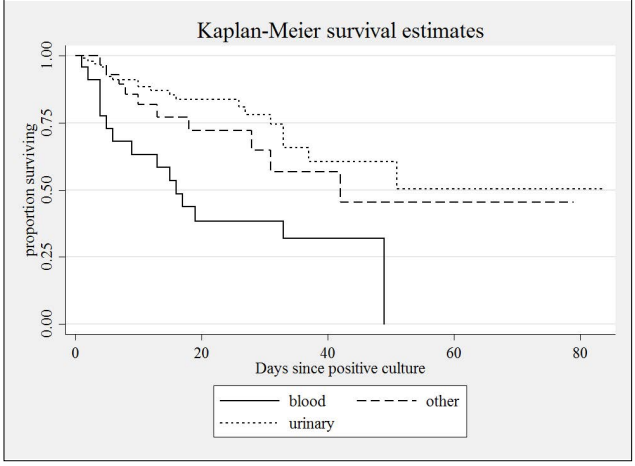
was 76.37 ± 12.99 years (range 26-96 years). The difference in age between males and females proved to be statistically significant (t = 2.6265, P < .01).
The mean duration of hospital stay was 35.32 ± 25.04 days (range 1-168).
In 46.28% of the cases of in-hospital infection, the infection was related to an invasive procedure (Tab. I).
The Age-adjusted *Charlson Comorbidity* Index revealed comorbidity values in the hospitalized patients of 6.21 and 6.67 in 2013 and 2014, respectively (P >.05).
Over the two-year observation period, 47.62% of the CRKP-infected patients were deemed at high risk. On considering the two years separately, a statistically significant difference emerged (X^2 = 16.0481, P < .001) between the percentage of patients classified as being at high infective risk in 2013 (32.00%) and in 2014 (63.89%).
A total of 70.77% of patients were hospitalized in 4-bed rooms, 17.69% in 2-bed rooms and 7.69% in single rooms. It was possible to hospitalize only 3.85% of patients in rooms with dedicated bathrooms (1.54% in 4-bed rooms, 1.54% in 2-bed rooms and 0.77% in single rooms).
Table I reports some characteristics of the cases of infection examined: the unit in which the diagnosis of infection was made, the provenance of the cases, the invasive procedures performed, the sites of infection and the specimen type.

Tab. II. Antibiogram for carbapenem-resistant *Klebsiella pneumoniae* cases reported.

Antimicrobial	No. resistant/No. tested	(%)
Aztreonam	145/147	(99)
Amikacin	45/55	(82)
Amoxicillin-clavulanic acid	145/147	(99)
Colistin	38/145	(26)
Cefalexine	91/92	(99)
Cefepime	102/147	(99)
Cefixime	91/92	(99)
Cefotaxime	145/147	(99)
Ceftazidime	145/147	(99)
Cefuroxime	144/145	(99)
Ciprofloxacin	145/147	(99)
Fosfomycin	17/130	(13)
Gentamicin	25/147	(17)
Levofloxacin	55/55	(100)
Moxifloxacin	91/92	(99)
Ampicillin	147/147	(100)
Piperacillin	55/55	(100)
Piperacillin-tazobactam	145/147	(99)
Tygecicline	5/41	(12)
Tobramycin	135/147	(92)
Thrimetoprim	76/94	(81)
Thrimetoprim-sulfamethoxazole	112/147	(76)

The incidence of infections was 0.442 pts/1000 days of hospitalization in 2013, and 0.513 pts/1000 days of hospitalization in 2014 (P > .05).
In the entire period of observation, we recorded 24 cases of coinfection due to: *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter baumannii, Enterococcus faecium, Proteus mirabilis, Corynebacterium striatum, Enterococcus casselliflavus, Moraxella morganii, Serratia marcescens,* and *Staphylococcus haemolyticus.* Among these microorganisms, we identified ESBL-positive strains of *E. faecium, E coli, P. mirabilis* and *P. aeruginosa*; VRE strains of *E. casseliflavus* and *E. faecalis*, and strains of MRSA.
With regard to patient outcomes over the two years of observation, 30.61% of the patients died in hospital; 12.24% were transferred to other units in the hospital; 0.68% were transferred to other hospitals; 19.73% were transferred to a residential facility; 4.76% were discharged with home assistance, and 31.97% were discharged home without assistance.
The in-hospital death rate was 24.0% in 2013 and 37.5% in 2014. However, the difference between these two values did not prove statistically significant (P > .05).
Analysis of mortality by means of Kaplan-Meier survival curves revealed that bloodstream infections were more frequently associated with death than were urinary infections and other infections (Fig. 1), the difference being statistically significant (X^2 = 14.57, P < .001).
Of the 23 cases of bloodsteam infections, 14 (12 hospital and 2 community) were cases of primary bacteremia; 10 of these patients died. The remaining 9 cases were secondary bacteremia, all nosocomial; 5 of these patients died. The mean age of the patients with primary bacteremia was 75.36 ± 10.70 years (range 58-90), while that of the patients with secondary bacteremia was 77.89 ± 9.57 years (range 63-92).
The age-adjusted Cox proportional hazard model revealed that the patients with bacteremia due to CRKP

Fig. 1. Kaplan-Meier curves of survival probability of patients with carbapenem-resistant *Klebsiella pneumoniae* infection, by infection site.



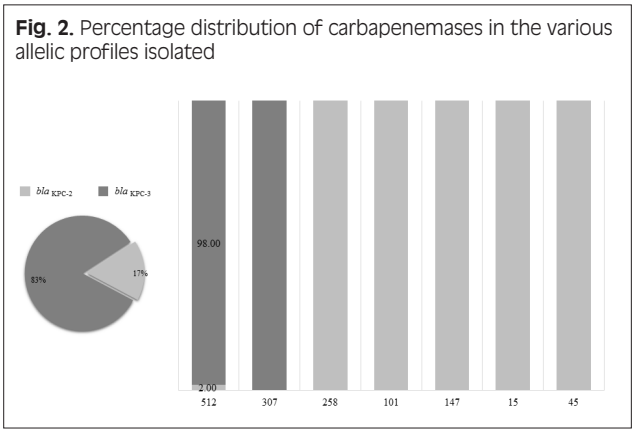


Fig. 2. Percentage distribution of carbapenemases in the various allelic profiles isolated

had a 3-fold higher risk of death (Hazard ratio [HR], 3.11; 95% CI 1.66-5.84, P < .001) than those with infections of other sites.

With regard to the results of antimicrobial susceptibility testing, the data on the resistance of the strains are reported in Table II; 26% of strains proved resistant to colistin. The antimicrobial ertapenem was the most frequently reported carbapenem tested for susceptibility (100%), followed by meropenem (98.6%) and imipenem (48.3%).

Genotyping of the strains by means of MLST revealed that the prevalent allelic profile was ST 512 (79.62%), followed by ST 307 (8.97%), ST 101 (3.85%), ST 147 (3.85%), ST 258 (3.85%), ST 15 (1.28%), and ST 45 (1.28%). Detection of the bla_{KPC} gene revealed that the most frequent carbapenemase was KPC-3 (83.8%) and that KPC-2 was less common (16.2%).

With regard to bla_{KPC} gene detection in relation to the allelic profiles, it emerged that STs 258, 101, 147, 15 and 45 displayed only bla_{KPC}-2; that ST 307 were associated only to bla_{KPC}-3; that most ST 512 hosted the bla_{KPC}-3 gene (98%), and that only a small portion hosted bla_{KPC}-2 (2%) (Fig. 2).

Discussion and conclusions

The emergence and spread of *Klebsiella pneumoniae* harboring carbapenemases have given rise to several problems regarding infection control and treatment.

Carbapenamase-associated resistance is alarming for a number of reasons. The presence of these enzymes, in addition to signifying resistance to carbapenems, is also associated with additional mechanisms of resistance to other antibiotic classes which, together, result in microbes that are highly multidrug resistant and in some cases panresistant [18-20]. Consequently, they are invariably associated with high treatment failure rates [21].

CRKP has rapidly become a major health concern for hospitalized patients in industrialized countries, and infection rates have been dramatically increasing worldwide over the past 10 years. The first case of carbapenemase-producing *Klebsiella pneumoniae* in Italy was detected in October 2008. In 2011, the European Antimicrobial Resistance Surveillance Network (EARS-Net) reported that Italy was one of the most seriously affected countries in Europe, with a worrisome increasing trend in CPKP [22-24].

These strains are implicated in nosocomial outbreaks and cause serious infections in ICUs. Moreover, recent national data have shown that CRKP is more frequently isolated from patients outside ICUs, and often from those admitted to geriatric or internal medicine wards [25, 26]. In the present study, we documented the occurrence of 147 cases of CRKP infections in a Northern Italian hospital. These cases were chiefly detected in medical wards (37.41%) and geriatric units (36.06%), followed by surgical wards (20.41%) and ICUs (6.12%). These wards are also frequently involved in CRKP infections in other countries. Indeed, Poulou et al. [27] reported that, of the 73 CRKP infections registered between 2009 and 2011 at a university hospital in Greece, 43.8% were identified in the ICU, 41.1% in medical wards and 15.1% in surgical wards. Moreover, Kanerva described an outbreak of carbapenemase-producing *Klebsiella pneumoniae* in a primary care hospital in Finland; this was confined to one geriatric ward and involved 142 patients with a mean age of 83 years [28].

Most of the CRKP-positive cases described in the present study involved elderly patients (mean age 78.95 ± 12.05 years). The Age-adjusted Charlson Comorbidity Index revealed values of underlying comorbidity in hospitalized patients of 6.21 in 2013 and 6.27 in 2014 (P > .05), reflecting a high level of complexity of assistance, which remained fairly constant throughout the observation period.

These findings are in line with those of recent studies, which have shown that the spread of CRKP in Italy is becoming a matter of concern in areas of care that were generally considered to be at lower risk. Moreover, one of the principal targets of CRKP is the population of geriatric patients [24, 29, 30], who display a high degree of clinical complexity and a large number of comorbidities and are frequently bedridden or cognitively impaired. Thus, frailty and comorbidity are, in themselves, a major risk factor for CRKP colonization, together with those already described in the literature (length of hospitalization, number of previous hospitalizations and/or previous ICU stays, previous antibiotic use, severity of illness, etc) [21, 30, 31].

In a large, retrospective, matched (1:2) case-control study in five Italian hospitals, Tumbarello et al. identified risk factors for CRKP infections; the strongest predictor of CRKP isolation was a history of ≥ 2 previous acute-care hospitalizations in the year before the index culture. Isolation was also associated with indwelling medical devices, such as urinary catheters, central venous catheters (CVCs) and surgical drains [21]. Invasive procedures are well-known risk factors for infection by CRKP [32]; indeed, the formation of biofilms on these devices is important in the pathogenesis of these bacteria [33, 34]. In 46.28% of the cases of in-hospital infection recorded in the present study, it was possible to correlate the infec-

tive event with an invasive procedure. Specifically, 75% of these cases occurred in patients with a urinary catheter; and indeed, the principal specimen type from which CRKP was isolated was catheter urine (44.22%). Lower percentages of infections related to invasive procedures were associated with CVCs (8.92%), endoscopic retrograde cholangiopancreatography (5.36%), and various other indwelling medical devices (overall, 10.72%).

During the two-year observation period, we registered an increase in the incidence of new clinical cases per 1,000 patient days. Fortunately, however, this increase did not reach statistical significance (0.442 cases/1,000 patient days in 2013 versus 0.513 cases/1,000 patient days in 2014; P>.05), despite the fact that a statistically significant difference ($\chi^2 = 16.0481$, $P < .001$) emerged between the percentages of patients classified as being at high infective risk in 2013 (32.00%) and in 2014 (63.89%).

Following laboratory identification of CRKP, the members of the Hospital Infections Committee implemented the interventions deemed necessary, with particular regard to the application of isolation measures, the disinfection of environmental surfaces and improvement of hand hygiene compliance. Indeed, eliminating surface contamination as a source of patient-to-patient transmission of nosocomial pathogens requires multiple interventions aimed at cleaning/disinfecting the environment and improving adherence to hand hygiene guidelines [35-37].

The mortality rate due to all causes of infection was 24% in 2013 and 37.5% in 2014. This increase, albeit not statistically significant, may have been due to the greater complexity of patients in the second year of observation. In any case, the mortality rates, however evaluated, proved to be in line with the literature data, which report mortality rates between 26% and 44 % [12, 38, 39].

Moreover, it emerged that the infective event most frequently associated with death was bacteremia; this is in agreement with the results of previous studies; in a matched retrospective, historical cohort design study involving 319 patients with infections due to carbapenem-resistant *K. pneumoniae*, Borer et al. [40] found a similar mortality risk ratio: 3.3 (95% CI 2.9-28.5) among case subjects with carbapenem-resistant *K. pneumoniae* bacteremia.

The results of our study revealed that 26% of strains proved resistant to colistin, a lower value than those reported in similar studies [41, 42].

Regarding the results of genotyping, it emerged that the allelic profile most frequently observed in the hospital was ST 512; this is in line with the results of other studies conducted in Italy [43]. ST 512 (allelic profile: 54-3-1-1-1-1-79) is a single-locus variant of ST 258 (allelic profile: 3-3-1-1-1-1-79) and is the clone most frequently associated worldwide with the spread of KPCs.

The ST 512 detected in the present study mainly produces KPC-3 carbapenemase; the bla_{KPC}-3-containing strain of *K. pneumoniae* displays an exceptional combination of multidrug resistance, virulence and ability to spread [44] and the KPC-3-producing *K. pneumoniae* ST 512 clone has emerged as a successful new lineage, capable of disseminating KPC-3 in Europe [45].

The results of the present study revealed that the characteristics of the predominant strain, together with the high levels of comorbidity of the patients involved and the difficulty of ensuring structural isolation owing to the small number of dedicated rooms, have, at least in part, undermined the success of the measures for prevention and control adopted in the hospital.

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Authors' contributions

MLC conceived, designed and coordinated the research. NC, PF and DU collected data.

DU and GO performed respectively microbiological and biomolecular analyses. ES performed the data quality control.

MS optimized the informatics database and performed the statistical analyses. MPC and GLP evaluated the results. MLC and AMS 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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ORIGINAL ARTICLE

Prevalence and antibiotic susceptibility profiles of *Listeria monocytogenes* contamination of chicken flocks and meat in Oyo State, south-western Nigeria: Public health implications

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Keywords

Listeria monocytogenes • Meat contamination • Public health

Summary

Introduction. *Food contamination with Listeria monocytogenes is on the increase posing threats to public health with growing trends in food products recalls due to suspected Listeria contamination.*
Methods. *We conducted a cross-sectional study to determine the prevalence and antibiotic susceptibility profiles of Listeria monocytogenes (Lm) among 71 randomly selected poultry farms in Oyo State, Nigeria. A total of 450 samples comprising cloacal swabs (426) and randomly selected dressed chicken meat (24) were cultured for Lm isolation using BrillianceTM Selective Listeria Agar with antibiotics and microbial load count with Nutrient Agar. Further identification was done using microscopic, biochemical characterization and antibiotic sensitivity tests. Data were analysed using bivariate analysis and student t-test.*
Results. *An overall prevalence of 91.8% Lm contamination was obtained comprising 91.5% (390/426) in cloacal swabs and 95.8% (23/24) in meat. The prevalence of Lm in cloacal samples was significantly associated with poultry type (p = 0.008) and breed (p = 0.000. In addition, all the flocks had at least one positive sample yielding 100% flock prevalence. Antibiotic sensitivity test revealed that most of the isolates were resistant to common antibiotics like Ampicillin-cloxacillin and cefuroxime.*
Conclusions. *The results revealed a high level of contamination with Lm in the poultry flock and meat and the observed resistance to most common antibiotics has implications for future disease control as well as public health. There is need to step up routine screening of food animal products for Listeria contamination as well as measures towards reducing such contaminations.*

Introduction

Listeria monocytogenes (Lm) is a facultative anaerobic bacterium which can grow and reproduce inside the host's cells, making it one of the most virulent food-borne pathogens. It belongs to the genus *Listeria*. *Listeria* spp. is widely distributed in environment. The genus consists of six species i.e., *Listeria monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri* and *L. grayi,* of which only *L. monocytogenes* is the primary human pathogen although there have been rare reports of illnesses caused by *L. seeligeri* and *L. ivanovii* [1-3]. *Listeria monocytogenes*, commonly referred to as *Listeria*, is a pathogen that causes listeriosis, a severe human illness [4, 5]. It is unlike most other food-borne pathogens because it can grow and multiply at proper refrigeration temperatures [6].
In addition, *Listeria* is widely distributed in nature [7], and has been recovered from farm fields, vegetables, animals and other environments such as surfaces of food processing facilities, retail stores and home kitchens and ready-to-eat foods [8-10]. *Listeria monocytogenes* represents a constant challenge for the food industry, health regulatory officials and consumers [11] since it remains one of the most virulent foodborne pathogens for immunodeficient individuals, It has been extensively studied over the past few decades due to its high case/fatality rate (20-30%), its high burden of healthcare costs during chronic episodes of infection and its ability to survive for longer periods under adverse environmental conditions than many other non-spore-forming bacteria [12]. In man, outbreaks usually occur following consumption of unpasteurized milk, contaminated cheeses and other dairy products. Reports of outbreaks have also followed ingestion of undercooked meat, poultry [13] as well as coleslaw where it was first recognized as a food-borne zoonosis [14]. It is frequently present in the gut of cattle, poultry and pigs and can be transmitted to ready-to-eat (RTE) foods as well as raw meat products [7]. *Listeria* species are isolated from a diversity of environmental sources, including decaying vegetation, soil, water, effluents, a large variety of foods, and the faeces of humans and animals [15]. Most reported isolations of this species were from abortions, stillbirths, and neonatal septicemias in sheep and cattle [16, 17].
Listeria monocytogenes is a major contaminant of RTE food and food products. Packaged raw foods can represent a potential source of contamination when opened at home, and listeriosis is associated with the consumption

of such undercooked raw foods [5]. Human to human transmission is rare, except in cases of pregnancy where infected mothers transmit the infection via the placenta to the unborn child. This results in abortion, still birth or death of newly-born infant [18]. Transmission in domestic animals can occur by ingestion of contaminated feed and poor quality silage with pH greater than 5.5, hence the name "silage disease" [19]. Outbreaks usually occur as septicaemia, meningoencephalitis (circling disease), and abortion.

There has been a dearth of information on the epidemiology of listeriosis in most African countries, including Nigeria [20] with only few reports, when compared to Europe and USA [21]. This is because the organism seems not to have been given attention as required. While antibiotic resistance has been reported severally in literature with clinical isolates from human beings, recent evidences however, show that antibiotic resistance traits have entered the microflora of farm animals and the food produced from them [22]. Thus, the food microflora is not separated from its human counterpart in cases of antibiotic resistance. The occurrence of antibiotic resistance complicates therapy and lengthens convalescence from illness [23]. This trend has been worsened by prophylactic use of common broad spectrum antibiotics, indiscriminate usage in humans and in animal feed as growth promoters, particularly in developing nations [23, 24]. Despite these and the increase in the consumption of poultry products coupled with enormous untrained hands in the poultry industry in Nigeria and the associated public health implications, there is paucity of information on the prevalence and antibiotic susceptibility profiles of *L. monocytogenes* among commercial chickens as well as raw processed chicken meat; hence, this study.

Methods

Study site, design, population and sampling

The study was carried out in 13 Local Government Areas (LGAs) known for the presence of high number poultry industries through a pilot survey across three Senatorial Districts of Oyo State, south-western Nigeria. The state was chosen as it possesses the majority of poultry industries in the region aside the backyard small scale poultry farming being practised by many. In addition, consumption of chicken and other poultry products is increasingly high in the state. This cross-sectional study involved a total of 71farms randomly selected from 100 available farms with different poultry types (layers, broilers), breeds, management (deep litter, battery cage) and biosecurity levels (high, average, low) located in the 13 LGAs of the state. The purpose of the study as well as the potential benefits was explained to the farm owners and they were told that participation was voluntary. It was also emphasized that declining participation did not have any attached penalty and that participation would not have any negative effects on their farms. The total number of poultry farms sampled was based on random selection of three of every four poultry farms through a transect walk guided by an initial pilot survey conducted. However, four of the selected farms declined participation. At each of the participating farms, cloacal swabs were collected using sterile swabs to scoop about one gram from each randomly selected chicken. 1ml of peptone water was then dispensed into each of the swab containers to moisten the samples in order to prevent the samples from drying up. Meat samples were also collected from points of retail into sterile sample bags. These were then placed in coolers containing ice packs for transportation to the Meat Hygiene Laboratory of the Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Nigeria for processing.

Microbiological analysis for *Listeria monocytogenes*

isolation was done using a slight modification of the methods described by Gibbons et al. [25] and Indrawattana et al. [26]. Peptone water was prepared by dissolving 15g of the powder in 1000mls distilled water and autoclaved at 121°C for 15min. Nutrient Agar was prepared by dissolving 28g of the powder in 1000mls of distilled water and autoclaved at 121°C for 15min. Listeria Selective Agar (LSA) (Brilliance™) was prepared by dissolving 33.6g of the powder base in 1000ml of distilled water, autoclaved for 15min at 121°C, cooled to 40°C and LSA antibiotics supplements was added. One gram of each sample was homogenized and transferred into a test tube containing sterile and freshly prepared peptone water. This was incubated at 37°C for 18 hours to 24 hours to revive viable but non-culturable cells. Thereafter, 100ul (0.1ml) each of the peptone water culture was transferred to a freshly prepared LSA and spread plated. Incubation was done at 37°C for 36-48 hours. Following incubation, discrete bacterial colonies were then counted from the incubated LSA for *Listeria monocytogenes* using the colony counter. Counts were transformed to colony forming unit (CFU) [27, 28]. *Listeria monocytogenes (Lm)* colonies appeared as green colonies with opaque white halos. Discrete *Lm* colonies from the LSA plates were then streaked onto freshly prepared LSA plates to obtain pure listeria isolates and the streaked plates were incubated at 37°C for 36-48 hours. Pure *Listeria monocytogenes* isolates were gram stained, then subjected to various morphological and biochemical tests which included catalase, oxidase and sugar fermentation using Glucose, Mannitol, Sucrose, Maltose, Fructose and Lactose. Phenolphtalein was used as indicator.

Assessment of the microbial load on samples screened

Serial dilution of each sample was also done up to the 6-fold dilutions, using freshly-prepared peptone water. 100ul (0.1ml) each of the 4th and 6th dilutions were then spread plated on nutrient agar plates and incubated at 37°C for 18-24 hours for counting. Following incubation, discrete bacterial colonies were then counted from the incubated nutrient agar plates using the colony coun-

ter. Counts were transformed to colony forming unit (CFU) and Log CFU.

Antibiotic susceptibility testing

This was performed using the Kirby-Bauer method (Disc diffusion Technique) [29]. The sensitivity discs were specifically designed and contained appropriate concentrations of different Gram positive antibiotics which include: ciprofoxacin (10µg/disc), norflaxacin (10µg/disc), gentamycin (10µg/disc), streptomycin (30µg/disc). Pure isolates were closely streaked onto the surface of Nutrient agar plates. The plates were then incubated at 37°C for 18-24 hours. Following incubation, they were observed for zones of inhibition surrounding each disc.

Data analysis

Data were analyzed using SPSS version 15. Chi-square test was used to test for association between the variables and prevalence of *Listeria monocytogenes*. Mean differences were analyzed using student's t-test (paired). Colonies counted were converted to colony forming units (CFU/ml). This was then transformed to base 10 Logarithms (CFU/ml). Mean standard deviation of CFU and Log10 CFU were calculated per sample type. Bacteria counts at the two different dilutions were compared among the sample types using paired t-test. The prevalence of *Listeria monocytogenes* contamination was calculated by dividing number of contaminated samples with the total number of samples collected. The epidemiological unit was the flock. A flock was considered contaminated by *Listeria monocytogenes* if at least one sample taken from the poultry house tested positive. The outcome variable "*Listeria monocytogenes* status" was dichotomous (contaminated (positive) versus non-contaminated (negative) flock). Prevalence was calculated based on the 100cfu/unit limit set by the European Commission Regulation (EC) No.2073/2005 on microbiological criteria for foodstuffs [30].

Results

Prevalence of *Listeria monocytogenes*

Of the 450 samples screened in this study, an overall prevalence of *Lm* contamination was found to be 91.8% comprising 95.8% (23/24) in meat and 91.5% (390/426) in cloacal swabs. All the flocks sampled had at least one positive sample yielding a flock prevalence of 100.0%. Cloacal samples from broilers had significantly higher prevalence (98.8%) than 89.8% from the layers (Tab. I). *Listeria monocytogenes* prevalence was highest among the Leghorn White (98.5%) and least among the Isa Brown breed (85.6%). Samples from poultry raised on deep litter (92.6%) and those from farms with low biosecurity level (93.2%) also recorded higher *Lm* prevalence. Overall, poultry type (X^2= 7.13; p =0.008); breed (X^2 = 15.25; p = 0.000), but not management (X^2 = 1.09; p = 0.297) as well as biosecurity level (X^2 = 0.173; p = 0.917) were significantly associated with the prevalence of *Lm* among the cloacal samples obtained (Tab. I).

Total bacteria count and antibiotic sensitivity test

Table II shows the comparison of bacteria counts (log CFU/ml) obtained at two different dilutions based on sample types. Mean bacteria counts obtained at 10^{-6} dilution were significantly higher (p = 0.0001) than those obtained at 10^{-4} dilution when compared across the sample type. The variations in mean logCFU/ml differences were significant across sample types (p = 0.0001).
A 100% resistance to both ampicillin-cloxacillin (30 ug) and cefuroxime (20 ug) antibiotics was demonstrated by the *Lm* isolates tested while the highest sensitivity

Tab. I. Occurrence of Listeria ***monocytogenes*** contamination based on poultry types, breed, management and biosecurity levels.

Variables	Category	Positive (%)	Negative (%)	Total	X^2; P value
Poultry type	Broilers	83 (98.8)	1 (1.2)	84	7.13; 0.008
	Layers	307 (89.8)	35 (10.2)	342	
Breed	Isa Brown	154 (85.6)	26 (14.4)	180	
	Nera Black	137 (95.1)	7 (4.9)	144	15.25; 0.000
	Leghorn White	65 (98.5)	1 (1.5)	66	
	Others*	34 (94.4)	2 (5.6)	36	
Management	Deep litter	261 (92.6)	21 (7.4)	282	1.09; 0.297
	Battery cage	129 (89.6)	15 (10.4)	144	
Biosecurity level	High	287 (91.4)	27 (8.6)	314	
	Average	62 (91.2)	6 (8.8)	68	0.173; 0.917
	Low	41 (93.2)	3 (6.8)	44	

*Harco Black, Anak White, Cobb USA

Tab. II. Total bacterial counts among the different samples taken (log CFU/ml).

Sample	1st Dilution(10^{-4})			2nd Dilution(10^{-6})			Paired t-test		
	Min	Max	Mean±SD	Min	Max	Mean ±SD	t	d_f	p-value
Cloaca	5.00	7.16	6.69± 0.25	7.00	9.00	8.43 ± 0.31	179.70	425	0.0001
Meat	6.41	7.15	6.71± 0.19	7.85	8.78	8.43 ± 0.25	44.67	23	0.0001

Tab. III. Antibiotic susceptibility of the *Listeria monocytogenes* isolates.

Antibiotics	Number of isolates tested	Amount sensitive	% sensitivity
Amocillin clavulanate(30ug)	72	62	86.1
Ciprofloxacin(10ug)	80	35	43.8
Cloxacillin(5ug)	72	26	36.1
Ceftriaxone(25ug)	80	26	32.5
Gentamicin sulphate(10ug)	72	20	27.8
Streptomycin sulphate (30ug)	80	20	25.0
Pefloxacin(10ug)	80	14	17.5
Erythromycin(5ug)	72	12	16.7
Co-trimoxazole(30ug)	88	11	12.5
Erythromycin(10ug)	72	9	12.5
Amoxacillin(30ug)	80	5	6.3
Ampicillin-cloxacillin(30ug)	80	0	0
Cefuroxime(20ug)	80	0	0

(86.1%) was obtained with amocillin clavulanate (30ug) (Tab. III).

Discussion

The overall high prevalence of 91.8% obtained in this study shows that *Listeria monocytogenes* is a common and constant contaminant of chicken flocks and chicken meat in the study area. This is similar to the findings of Gaffa & Ayo [31] and Chukwu et al. [32] in ready-to-eat (RTE) dairy products; and Nwachukwu et al., [33] in *Kunu*. Our findings further corroborate previous reports that *Listeria monocytogenes* is an important food-borne pathogen and is widely distributed in food, environmental and clinical samples [2, 34, 35]. As observed from our findings, the meat samples had higher incidence of *L. monocytogenes* (95.8%) when compared to cloacal samples (91.5%). These higher counts in meat could have resulted from the unhygienic handling practices of meat handlers and processors. As reported, contamination usually arises from unwholesome contacts of meat with excretions from skin, mouth and nose of the meat processors [36, 37]. It also suggests likely cross-contamination of raw processed chicken by improperly cleaned and disinfected processing environment and to a lesser degree from the live chicken. This finding concurs with similar findings by Cox et al. [38] and Kanarat et al. [39] which put processing as a major hazard of cross-contamination. The very high prevalence in raw processed chicken meat samples in this study is similar to the report by Gibbons et al. [25] which indicated 90.9% prevalence in raw meat. These findings coupled with poor food handling practices in the study area therefore portends serious health hazards to the public considering possible contamination with other raw food items during food preparation.

Comparatively, most Listeria cases are reported in high-income countries, while cases are much more likely to go unreported in developing countries. Most cases of listeriosis are sporadic and have been reported in high-income countries, where incidence is quite low but fatality rate is high [40]. Recently, Effimia [41] reported a 14.4% prevalence of *L. monocytogenes* in ready-to-eat food products in Greece while Wu et al. [42] observed a 20% prevalence in retail foods in China. Important outbreaks have also occurred-for example, an outbreak of listeriosis from cantaloupes in Colorado, USA, in 2011 resulted in infection of 147 people and 33 deaths, making it the deadliest recorded US foodborne outbreak since the US Centers for Disease Control and Prevention (CDC) began tracking outbreaks in the 1970s [43-44]. Listeriosis often results in admission to intensive-care units, which makes *L. monocytogenes* the third most costly foodborne pathogen in the USA per case in 2010, after *Clostridium botulinum* and *Vibrio vulnificus* [45]. Ivanek and colleagues [46] estimated that the annual cost of *L. monocytogenes* in the USA was US$2·3 billion to 22 billion, and the annual benefit of listeria food safety measures was $0·01 billion to 2·4 billion.

Our findings also observed a higher *Lm* prevalence among poultry flocks on deep litter than those in battery cage system. A previous report indicated that *Lm* can survive and multiply in wet litter [47] and thus serves as a source of contamination to the poultry flock. This may also explain the higher *Lm* prevalence recorded among broilers than layers in this study since broilers were in most cases raised on deep litter system. Litter should therefore be regularly changed and be protected from moisture. Also, it should always be stored in an enclosed location in order to protect it from pests such as wild bird so as to avoid contamination by wild life.

Similarly, the results of this study also suggest a significant association between the breeds of poultry flocks and *Lm* prevalence, with the Isa Brown breed showing the least prevalence. This could be as a result of possible varying resistance associated with different breed types. A further research into the genetic variations of breeds of poultry with reference to resistance/susceptibility to disease organism is required. On the other hand, while there was no statistically significant association between *Lm* prevalence and biosecurity levels of the different

farms, *Lm* prevalence was highest in farms with low biosecurity level. Previous studies have also showed that farms with low biosecurity level have increased risk of *Lm* contamination [47, 48].

In addition, most of the *Listeria monocytogenes* isolates obtained in this study showed profound resistance to the majority of the common antibiotics with 100% resistance to ampicillin cloxacillin and cefuroxime. This observation suggests a gross antibiotic abuse among poultry farmers in the study area. A similar report was previously made by Adetunji and Ishola [49] and Nwachukwu et al. [33] who revealed a profound resistance to Ampicillin , which is the drug of choice for treating listeriosis. It was, however in contrast to the report by David and Odeyemi [50] who found that broad-spectrum drugs like chloramphenicol and fluoroquinolones were significantly effective against this organism. Again, the susceptibility of most of the *Listeria monocytogenes* to gentamicin sulphate in this study is similar to previous reports [51, 52] which indicated susceptibility of all the *L. monocytogenes* obtained to this antimicrobial agent. The susceptibility of most of the *L. monocytogenes* in this study and previous studies to gentamicin sulphate plausibly suggests that this antimicrobial remains an alternative regimen against the organism. Given the multiple resistance shown by the *L. monocytogenes* to antimicrobial agents, the implication could be that the cost of treatment will be very high when humans are infected with these zoonotic pathogens; assertions which are in agreement with other reports [52, 53].

Similarly, the high incidence of *Listeria monocytogenes* in cloacal samples (64.8%) may be attributed to the constant ingestion of listeria-contaminated feed and water. This is similar to findings by Schlech et al. [14] and Gravani [34] which stated that listeria are mainly found in soil, silage and water. Though, the gut of birds is a usual habitat for *Listeria monocytogenes* [54]; Skovgaard [55] and Larpent [56] reported a common occurrence of *Liseria monocytogenes* in animal feaces.

Despite the high prevalence of *Listeria monocytogenes* in this study, most of the chickens showed no sign of infection. This further reiterates the claim by Cox et al. [38], that chickens are faecal carriers of the organism and may contaminate the litter and environment of the poultry house. Also, there seemed to be no significant increase in *Lm* counts with total microbial load, as samples with highest *Lm* counts did not necessarily have the highest microbial load, and vice-versa. This could be explained by the fact that *Lm* is very hardy and persists in the environment, resisting most cleaning and disinfectant techniques, unlike many other bacteria which are eliminated by cleaning and disinfecting [49, 57].

Conclusions

This study showed a high overall incidence (91.8%) of *Listeria monocytogenes* in poultry flocks and poultry meat in Oyo state, Nigeria. The higher incidence in meat suggests post-slaughter contamination and portends health hazards to the public through contact between these raw meat and other processed foods. It also shows that poultry flock types and breeds were significant factors associated with *Lm* contamination. In addition, the resistance of *Listeria monocytogenes* isolates to most of the antibiotics in this study is a matter of concern both to the future management of poultry diseases as well as public health. We therefore recommend that farm –to-fork principles of hygiene should be stepped up particularly among poultry and other food handlers in order to limit contamination with food pathogens. Standard Operating Procedures (SOP) and Hazard analysis and critical control points (HACCP) should be developed and implemented by poultry regulatory agencies such as Poultry Association of Nigeria (PAN) and Poultry Farmers of Nigeria (PFN). Government should enforce prompt registration and periodic monitoring of all poultry farms and abattoirs in order to institute measures to check the sanitary levels of farms and abattoirs and enforce strict adherence to hygiene standards on a continual basis. Farmers should be enlightened on appropriate antibiotic usage and withdrawal period.

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Authors' contributions

OOI developed the concept of the study and wrote the manuscript; JIM did the sample collection and analysis and was involved in the writing of the manuscript; HKA did the statistical analysis of the data and was involved in the writing of the manuscript.

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ORIGINAL ARTICLE

Prevalence and predictors of risk factors for Brucellosis transmission by meat handlers and traditional healers' risk practices in Ibadan, Nigeria

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Keywords

Brucellosis • Public health • Risk factors

Summary

Introduction. *Brucellosis is endemic in Nigeria and risk factors enhancing its transmission are prevalent.*
Methods. *Following serological evidence of brucellosis and isolation of B. abortus from slaughtered cattle in Ibadan, Nigeria, we administered a semi-structured questionnaire to determine the prevalence and predictors of eating and selling bovine gravid uterus among 350 meat handlers from five major meat processing facilities. We conducted key informant interview for five leading traditional healers to document its use. Data were analyzed using Stata 12.*
Results. *The prevalence of eating and selling gravid uterus were 29.7% and 40.3% respectively. Being meat/offal processor (OR=1.9, 95%CI: 1.11-3.3, P = 0.008) and not knowing that eating undercooked contaminated gravid uterus could expose humans to brucellosis (OR=19.5; 95%CI: 5.73-66.03; P = 0.000) were strong predictors of eating gravid uterus. Similarly, being adult (OR = 1.7, 95%CI: 1.08-2.57, P = 0.02) and inadequate knowledge of brucellosis as a preventable disease (OR = 0.03; 95%CI: 0.004-0.27, P = 0.001) predicted selling gravid uterus. Qualitative data from the traditional healers revealed using gravid uterus as special medicinal preparations to hasten parturition in overdue pregnancies, treat infertility and old age diseases in humans.*
Conclusions. *We demonstrated a high prevalence of risk factors for brucellosis transmission, and some meat handlers' socio-demographic characteristics and brucellosis knowledge-based markers as predictors of these factors. The traditional healers' practices portend a challenge to the current brucellosis control strategy. These findings provide insights into designing all-inclusive health programmes aimed at controlling brucellosis spread in Nigeria and other similar settings in developing countries.*

Introduction

Brucellosis is one of the most important zoonoses in the world [1]. The disease is endemic in many regions of the world, including Latin America, the Middle East, Africa, Asia and the Mediterranean basin [2]. The global burden of the disease in humans remains enormous with more than 500,000 infections per year worldwide [2-4]. It has been reported as an important cause of Fever of Unknown Origin [5] and particularly among the occupationally exposed groups, [6] as it is often easily misdiagnosed as other febrile syndromes such as malaria and typhoid fever, thereby resulting in mistreatments and underreporting [7]. Meanwhile, all brucellosis infections in humans are due to direct or indirect contact with infected animals or animal materials [8] and the incidence is directly related to the prevalence of the disease in animals, socioeconomic level, eating habits, poor hygiene and practices that expose humans to infected animals or their products [9]. It is acquired in people through breaks in the skin following direct contact with infected animals' tissues or blood or their secretions. Infection may also result from consumption of contaminated unpasteurised milk and milk products [10] as well as undercooked contaminated meat [11, 12].

Human brucellosis is widespread in Nigeria, particularly among the occupationally exposed groups. In the North-Eastern part of the country, Baba et al. [13] reported a 5.2% prevalence of brucellosis among 500 occupationally exposed patients. In another study in North-Central part of the country, 43.8% of the 7.8% brucellosis infected hospital patients were abattoir workers [14]. In addition, Aworh et al. [15] documented a 24.1% seroprevalence of brucellosis among abattoir workers at the Federal Capital Territory, Abuja, Nigeria. Over 55% of 7161 people examined in different parts of western Nigeria had positive *Brucella abortus* antibodies in their sera, with higher incidences of titres found among dairy farmers and slaughter men than the general population [16].

Specifically, continuous evidences of serological prevalence of brucellosis among the slaughtered cattle in Ibadan, South-Western Nigeria abound ranging between 5.31 and 8.6% [17-20]. In humans, Cadmus et al. [18] reported a high seroprevalence of 66.3% of brucellosis among apparently healthy abattoir workers while recent unpublished data confirmed isolation of *B. abortus* from slaughtered cattle in the same area. Despite these, the

practice of eating and selling gravid uterus is common among meat handlers. In addition, traditional healers reportedly make use of gravid uterus locally called *abodi alaka* for some concoctions; whereas, a gravid uterus sustains the growth of *Brucella* organism [21, 22]. The risk is potentiated by the habit of eating uncooked or undercooked meat as well as poor handling during food preparation [11, 23]. This study was aimed at determining the prevalence and predictors of the risk behaviours of eating and selling gravid uterus by meat handlers and also documenting usage of this organ by leading traditionalists in Ibadan, Nigeria.

Materials and methods

Study Design, Site and Population

This cross-sectional study was conducted in Ibadan, Nigeria. Nigeria is the most populous country in Africa (over 170 million in 2012; http://esa.un.org/wpp/ASCII-Data/DISK_ NAVIGATION_ASCII.htm) with an estimated livestock population of 20.49 million cattle, 23.07 million sheep, 28.07 million goats, 6.54 million pigs (http://www.fao.org/ag/againfo/ resources/en/glw/GLW_dens.html),18,200-90,000 camels, and 210,000 horses (http://faostat.fao.org/site/573/default.aspx#ancor) [24]. It ranks second of the four countries (Nigeria, India, Ethiopia, and Bangladesh) that account for 44% of poor livestock keepers globally [25]. Ibadan is located in South-Western Nigeria and lies between latitude $7^{0}32^{1}$N and longitude $3^{0}54^{1}$E. It is the third largest metropolitan area, by population, as well as the largest metropolitan geographical area in the country. Previous and on-going reports showing serological evidence of brucellosis [17-20] as well as isolation of *B. abortus* (unpublished data) in slaughtered cattle in this study area abound. The study was carried out using the five major government-owned meat processing facilities which supply meat to the teeming population of over 2 893 137 people [26] in the area, including its surrounding environments. These meat processing facilities were chosen on the basis of the populations of their workers (Oyo State Department of Agriculture and Rural Development, personal communication) while the food animals slaughtered represent more than 65% of the slaughtered animals in the area.

The study spanned a period of two months. The population at the meat processing facilities from which the respondents were selected consisted of meat butchers, meat/offal processors, meat buyers and children. The inclusion criteria for selection of potential participants were being meat handlers actively participating in meat processing operations and being at least 18 years of age. A meeting was held with all the potential participants on the objectives and benefits of the study and were informed that they could choose either to participate or not in the study. They were then grouped based on the slaughter halls where each of them worked. A pretest was conducted among ten randomly selected meat handlers, after which some of the questions were modified to improve clarity. Thereafter, visits were made based on the groupings and all consenting participants who met the inclusion criteria, excluding those who participated in the pretest, were interviewed. Each of them was allotted a code on the questionnaire. The researchers made provisions for interpreters for those who did not understand English, but only their local language. In all, only 17 people among those who met the inclusion criteria and were asked to be interviewed declined participation. In addition, the researchers identified a key leader who was knowledgeable about the traditional settings in each of the areas where the meat processing facilities used were located. These key leaders assisted the researchers in identifying the leading traditional healers in the areas for interview.

Data collection and analysis

Data for this study on the participating meat handlers were collected using a semi-structured interviewer-administered questionnaire by well-trained personnel. The questionnaire included three parts. In the first part, we attempted to determine the socio-demographic profiles of the respondents including the age groups (18-40 years as young adult and > 40 years as adult), sex, highest education received, nature of occupation and length of years already spent as workers in meat processing. The second part had five questions to determine their knowledge on bovine brucellosis as it relates to its transmission to humans with response options of 'yes', 'no' or 'I don't know'. The third part contained five questions inquiring about their risk behaviours including whether or not they eat, or sell gravid uterus with response options of either 'yes' or 'no'. Using a key informant interview, the identified leading traditional healers were asked questions on their uses of gravid uterus as well as on issues related to their awareness and knowledge of brucellosis transmission with respect to their practices. Their responses were documented, collated and summarized.

The central study outcome variables from the questionnaires on the meat handlers were whether the respondents did or did not eat or sell gravid uterus and those who indicated eating or selling it were classified as high risk and those who did not as low risk. The independent variables were demographic variables and knowledge-based markers related to brucellosis. Data were analyzed using Stata 12.0 (StataCorp LP, Texas, USA) and were tabulated based on the risk category. The values in each category were presented together with their respective percentages. Univariate analysis was first done on all variables using chi-squared statistic with Fisher's exact test when necessary to determine potential variables for the logistic regression model. A multivariate unconditional logistic regression analysis was done using the variables that were statistically significant at 10% level. Backwards stepwise regression was used with the least significant variable removed at each stage until the model contained only those factors which were significant at the 5% level. All tests were two-tailed and p-values of less than or equal to 5% were considered significant.

The odds ratios were reported with their 95% confidence intervals (CI).

Results

A total of 350 meat handlers and five leading traditional healers participated in this study. Out of these meat handlers, 104 (29.7%) and 141 (40.3%), respectively affirmed eating and selling gravid uterus, thereby constituting the high risk groups (Tab. I). Based on socio-demographic characteristics, 50.9% were young adults, 62.9% were male respondents, 52.3% had primary education, 57.1% were meat/offal processors and 64.0% had been in meat processing facilities as workers for more than ten years (Tab. II).

Assessment of predictors of eating gravid uterus by meat handlers

Of all the socio-demographic variables, only being meat/offal processors (P = 0.008) was the significant factor associated with eating gravid uterus. The meat/offal processors (OR: 1.9, 95% CI: 1.11-3.30) respondents were about two times more likely to eat gravid uterus than the butchers (Table II). Furthermore, the low risk group (those who did not eat gravid uterus) demonstrated significantly better knowledge than those who ate gravid uterus. For instance, 18.3% of the low risk group and only 1% of the high risk group knew that *Brucella*-contaminated gravid uterus could contaminate other raw meat or food materials by contact (P = 0.000). Again, 38.2% of the low risk group and only 2.9% of the high risk group knew that consumption of under-cooked or raw contaminated gravid uterus could expose humans to infection with brucellosis (P = 0.000). However, the two groups did not differ significantly (though the low risk group demonstrated higher knowledge level) in whether or not brucellosis was a preventable disease (P = 0.322) (Table III). Overall, not knowing that consumption of undercooked or raw contaminated gravid uterus could expose humans to brucellosis (OR = 19.5, 95%CI: 5.73-66.03, P =0.000) and that it could contaminate other food materials or raw meat (OR = 15.6, 2.05-118.92, P =0.008) were the strong predictors of eating gravid uterus by the meat handlers. Lower risks of eating gravid uterus were predicted by having heard of brucellosis and knowing brucellosis as a zoonosis (Tab. III).

Assessment of predictors of selling gravid uterus by meat handlers

Only being adult (OR: 1.7, 95% CI: 1.08-2.57, P = 0.02) of all the socio-demographic variables examined was the strong predictor of selling gravid uterus by meat handlers,

Tab. I. Prevalence of risk factors for brucellosis transmission to humans among meat handlers in Ibadan, Nigeria (n = 350).

Variable	N (%; 95% CI)
Eat gravid uterus	104 (29.7; CI: 24.9 - 34.5)
Sell gravid uterus to unsuspecting buyers as some other meat parts	141 (40.3; CI: 35.2 - 45.4)
Do not wear protective coverings when handling gravid uterus	289 (82.6; CI: 78.6 - 86.6)
Do not separate gravid uterus from other raw meat	131 (37.4; CI: 32.3 - 42.5)
Do not wash hands after handling gravid uterus	215 (61.4; CI: 56.3 - 66.5)

Tab. II. Socio-demographic characteristics of meat handlers in relation to the risk factor of eating gravid uterus in Ibadan, Nigeria (n = 350).

Variable	Category	Total n (%)	Do not eat gravid uterus (n = 246) %	Eat gravid uterus (n = 104) %	Univariate *P*-value	Logistic regression OR, 95% CI, *P*-value
Age	Young adult	178 (50.9)	50.4	51.9	0.80	NA*
	Adult	172 (49.1)	49.6	48.1		
Gender	Male	220 (62.9)	61.4	66.3	0.38	NA*
	Female	130 (37.1)	38.6	33.7		
Education	None	60 (17.1)	15.4	21.2	0.39	NA*
	Primary	183 (52.3)	54.1	48.1		
	Post-primary	107 (30.6)	30.5	30.8		
Duration in meat processing facilities (in years)	≤ 10	126 (36.0)	35.4	37.5	0.70	NA*
	> 10	224 (64.0)	64.6	62.5		
Occupation	Butchering	150 (42.9)	45.5	36.5	0.02	
	Meat/offal processing	200 (57.1)	54.5	63.5		1.9, 1.11-3.30, 0.008

*NA: Variables not significant at univariate analysis and were not included for logistic regression.

Tab. III. Knowledge levels of brucellosis by meat handlers in Ibadan, Nigeria with respect to risk category (n = 350).

Variable	Total n (%)	Do not eat gravid uterus (n = 246) %	Eat gravid uterus (n = 104) %	Univariate *P*-value	Logistic regression OR, 95% CI, *P*-value
Have you heard of brucellosis?					
Yes	14 (4.0)	4.1	3.8		
No	336 (96.0)	95.9	96.2	0.015	0.2, 0.04-0.71, 0.016
Does brucellosis spread from animals to man?					
Yes	9 (2.6)	3.7	0.0		
No	111 (31.7)	23.2	51.9		7.3; 0.89-60.42; 0.065
I don't know	230 (65.7)	73.1	48.1	0.000	2.2; 0.27-18.19; 0.457
Does *Brucella*-contaminated gravid uterus contaminate other food material/raw meat by contact?					
Yes	46 (13.1)	18.3	1.0		
No	101 (28.9)	30.5	25.0		15.6; 2.05-118.92; 0.008
I don't know	203 (58.0)	51.2	74.0	0.000	27.5; 3.72-203-57; 0.001
Does consumption of under-cooked or raw contaminated gravid uterus expose humans to brucellosis infection?					
Yes	97 (27.7)	38.2	2.9		
No	94 (26.9)	23.6	34.6		19.5; 5.73-66.03; 0.000
I don't know	159 (45.4)	38.2	62.5	0.000	21.7; 6.58-71.38; 0.000
Is brucellosis a preventable disease?					
Yes	14 (4.0)	4.9	1.9		
No	76 (21.7)	20.3	25.0		
I don't know	260 (74.3)	74.8	73.1	0.322	NA*

*NA: Variables not significant at univariate analysis and were not included for logistic regression.

with the adult respondents being almost two times more likely to sell gravid uterus than the young adult group (Tab. IV). With respect to knowledge-based markers for brucellosis transmission, the low risk group demonstrated significantly better knowledge than the high risk group, except on the questions that related to whether or not consumption of contaminated gravid uterus could expose humans to brucellosis as well as whether brucellosis was a preventable disease or not (Table V). In all, lower risks of selling gravid uterus were predicted by knowing that consumption of contaminated gravid uterus could expose humans to brucellosis infection (OR = 0.2, 95%CI: 0.13-0.44, $P = 0.000$) and that brucellosis was a preventable disease (OR = 00.3, 95%CI: 0.004-0.27, $P = 0.001$) (Tab. V).

Qualitative data from traditional healers on the usage of gravid uterus

Qualitative data from the leading traditional healers' key informant interview revealed high risk behaviour for brucellosis transmission. Responding to the question on what they used gravid uterus for, they said "*We usually use it to treat some health conditions associated with old age, to hasten parturition in overdue pregnancies as well as to treat infertility in women*". According to them, gravid uterus was made into special medicinal preparations for the affected individuals to eat. However, none of the traditional healers knew any animal disease that could be associated with gravid uterus neither were they aware of the possibility of brucellosis transmission from eating contaminated gravid uterus.

Discussion

The global burden of human brucellosis remains enormous [2, 4]. Though eradicated in many developed countries after years of effort, the disease is still a major neglected zoonosis of developing countries, including Nigeria [1]. The incidence is directly related to the prevalence of the disease in animals, eating habits, poor hygiene and practices that expose humans to infected animals or their products [9]. As such, livestock workers, including meat handlers, have been incriminated in the spread of human brucellosis in Nigeria [15, 27-28]. Poor hygiene and eating of raw or improperly cooked contaminated meat, the practices characteristic of meat handlers in Nigeria are known to favour the spread of brucellosis [11-12]. In order to reduce the spread of human brucellosis in the country, knowledge about the predictors of the risk factors of eating and selling gravid uterus known to sustain *Brucella* organisms is essentially required. This current study presents the socio-demo-

Tab. IV. Socio-demographic characteristics of meat handlers in relation to the risk factor of selling gravid uterus in Ibadan, Nigeria (n = 350).

Variable	Category	Total n (%)	Do not sell gravid uterus (n=209) %	Sell gravid uterus (n=141) %	Univariate *P*-value	Logistic regression OR, 95% CI, *P*-value
Age	Young adult	178 (50.9)	56.0	43.3	0.02	
	Adult	172 (49.1)	44.0	56.7		1.7; 1.08-2.57; 0.02
Gender	Male	220 (62.9)	66.5	57.5	0.085	1.5; 0.95-2.29, 0.086
	Female	130 (37.1)	33.5	42.6		
Education	None	60 (17.1)	17.2	17.0		
	Primary	183 (52.3)	50.7	54.6	0.733	NA*
	Post-primary	107 (30.6)	32.1	28.4		
Duration in meat processing facilities (in years)	≤ 10	126 (36.0)	38.8	31.9	0.191	NA*
	>10	224 (64.0)	61.2	68.1		
Occupation	Butchering	150 (42.9)	44.5	40.4	0.45	NA*
	Meat/offal processing	200 (57.1)	55.5	59.6		

*NA: Variables not significant at univariate analysis and were not included for logistic regression.

Tab. V. Knowledge levels of bovine brucellosis by meat handlers in Ibadan, Nigeria with respect to risk category of selling gravid uterus (n = 350).

Variable	Total n (%)	Do not sell gravid uterus (n = 209) %	Sell gravid uterus (n = 141) %	Univariate *P*-value	Logistic regression OR, 95% CI, *P*-value
Have you heard of brucellosis?					
Yes	14 (4.0)	4.8	2.8		
No	336 (96.0)	95.2	97.2	0.362	NA*
Does brucellosis spread from animals to man?					
Yes	9 (2.6)	4.3	0.7		
No	111 (31.7)	39.2	19.9		2.7; 0.32-22.54; 0.359
I don't know	230 (65.7)	56.5	79.4	0.000	7.6; 0.94-61.69; 0.058
Does *Brucella*-contaminated gravid uterus contaminate other food material/raw meat by contact?					
Yes	46 (13.1)	13.9	12.1		
No	101 (28.9)	22.5	38.3		2.0; 0.96-4.01; 0.065
I don't know	203 (58.0)	63.6	49.7	0.006	0.9; 0.46-1.75; 0.751
Does consumption of under-cooked or raw contaminated gravid uterus expose humans to brucellosis infection?					
Yes	97 (27.7)	15.8	45.4		
No	94 (26.9)	30.6	21.3		0.2; 0.13-0.44; 0.000
I don't know	159 (45.4)	53.6	33.3	0.000	0.2; 0.13-0.37; 0.000
Is brucellosis a preventable disease?					
Yes	14 (4.0)	0.5	9.2		0.03; 0.004-0.27;
No	76 (21.7)	25.4	16.3		0.001
I don't know	260 (74.3)	74.2	74.5	0.000	0.1; 0.01-0.40; 0.005

*NA: Variables not significant at univariate analysis and were not included for logistic regression.

graphic factors of meat handlers and brucellosis knowledge-based markers which influence the occurrence of the risky practices of eating and selling gravid uterus in Nigeria. It also reports the implications of traditional healers' usage of gravid uterus on the epidemiology and control of human brucellosis in the country.

To our knowledge, this study appears to be the first to investigate the predictors of the risk factors of eating and selling gravid uterus by meat handlers in Nigeria as well as traditional healers' practices in relation to brucellosis transmission. This study has established a high prevalence of risk factors for human brucellosis infection including the primary outcomes of interest, namely eating and sell-

ing gravid uterus by meat handlers. These include: eating gravid uterus (29.7%); selling gravid uterus to unsuspecting buyers (40.3%); not wearing protective coverings when handling gravid uterus (82.6%), not washing hands after handling gravid uterus (61.4%) and not separating gravid uterus from other meat parts (37.4%).

Our findings showed that almost one-thirds and above two-fifths of the meat handlers, respectively engaged in eating and selling gravid uterus. These practices by this high risk occupational group are a matter of public health concern considering the prevailing serological evidences of brucellosis and reported isolation of *B. abortus* from the same population of slaughtered cattle (unpublished data) in the study area. The ingestion of tissues, foodstuff or fluid containing *Brucella* organism is a route of brucellosis transmission [11]. As such, there is a high risk for human infection with brucellosis among these meat handlers and other potential consumers who are exposed, given the habit of eating raw or improperly cooked meat which is common amongst livestock keepers and meat handlers in Nigeria [28, 29] and amongst Africans in general [11, 12]. In addition, poor hygienic practices characteristic of meat handlers and most households in developing countries, including Nigeria [30, 31], could as well enhance the transmission of the organism. As reported, handling and preparation of infected meat and offal without proper hygienic precautions may lead to contamination of other foods [32]. Similarly, while brucellosis is a worldwide known abortifacient disease [33] and an important cause of infertility in infected animals [34], routine use of gravid uterus from brucellosis endemic cattle population by the traditional healers in treating health conditions associated with old age, overdue pregnancies and infertility is startling. The need to investigate indigenous or traditional handling of animals and animal products in the epidemiology of human diseases, including brucellosis in Nigeria and other developing countries, becomes apparent.

Evaluation of demographic variables showed that at least one or more of being adult respondents and meat/offal processors were significantly associated with the high risk factors of eating and selling gravid uterus by the meat handlers. In this study, although not statistically significant, the male respondents were about two times more likely to sell gravid uterus than the female respondents. This finding is in agreement with the reports of some other workers with respect to risk taking by the male respondents. Hambolu et al. [29] observed that being male respondents was an important predictor of the high risk behaviour of consumption of *fuku elegusi* (tuberculosis-infected lungs) amongst abattoir workers in Nigeria. According to Courtenay [35] and Davidson et al. [36], predominance of risk-taking amongst male humans is inherently related to the social construction of masculinity. In addition, male subjects are more involved in the care and management of animals as well as processing of meat than the female subjects; hence, they are likely to be more involved in risk practices associated with the occupation. Again, the report of European Commission [37] on risk taking in food handling indicated that women seem to be somewhat more susceptible to worry when it comes to the risk perceptions. This explains whythey have a lower tendency to be involved in taking risks. Other studies [38-39] have also consistently shown men to have less than ideal food hygiene practices and a significantly lower knowledge of food safety issues.

Furthermore, the adult meat handlers were about twice more likely to be involved in the sale of gravid uterus than the younger age group. Adult meat handlers have been reported to exhibit lower food safety practices [31]. Likewise, Altekruse et al. [40] reported that unsafe practices were reported more often by men and adults. This occurrence among the adult meat handlers might be associated with the observation that they often feel unconcerned with any possible consequences that could be associated with such risky practices for lack of evidence-based immediate effects on them. And since brucellosis mimics other febrile conditions and could be latent for years [41], they always tend to equate any feverish conditions they experience to either malaria or typhoid fever.

The study also showed that meat/offal processors were twice more likely to eat gravid uterus than the butchers. The reason for this might be because the meat/offal processors generally have more direct contact with gravid uterus considering the nature of their work. Generally, offal processors have a more constant contact with viscera, gravid uterus and fetal membranes of infected animals (the preferred sites of localization of the bacteria) and are generally more prone to contract brucellosis [42, 43]. Hence, there is a higher likelihood of eating the products they often deal in than the butchers who only have occasional contact since the offal processors end up processing all the viscera/offals from various slaughtered animals.

Although the low risk group exhibited better knowledge levels than the high risk group, it is disheartening that the knowledge levels of the entire population on issues related to brucellosis, its transmission and prevention were far below average. Ordinarily, one would expect people drawn from such a high risk occupation to be prioritized with messages regarding brucellosis. This poor knowledge as well as the risky practices coupled with the endemicity of bovine brucellosis in cattle population [44, 45] in Nigeria is a matter of public health concern. This is evident by the high seroprevalence of human brucellosis reported amongst livestock workers in Ibadan, South-Western Nigeria [18]. Reports from Tanzania also showed highest seroprevalence of brucellosis amongst abattoir workers, particularly those involved in the slaughtering and cleaning of slaughtered animal parts [46] and a 48% seroprevalence amongst families associated with livestock keeping [12]. Alavi et al. [47] also reported an association between work practices and *Brucella* infections amongst nomads in Khuzestan, Iran.

Our findings notwithstanding; one limitation of this study is the use of only government-owned meat processing facilities. Inclusion of private meat facilities could have

given more comprehensive insights. However, the findings of this study are generalizable to meat handlers in Nigeria as the chosen facilities are typical of other meat processing facilities in terms of conditions of the facilities and the ways by which meat handlers are regulated. Despite this limitation, the study has demonstrated a high prevalence of risk factors for human brucellosis transmission as well as some socio-demographic characteristics of meat handlers and knowledge-based markers as predictors of risk factors of eating and selling gravid uterus in Ibadan, Nigeria. It has also reported risk practices by traditional healers that could serve as a limiting factor to brucellosis control in the area. The information provided are very important insights in understanding the epidemiology of human brucellosis in Nigeria and thus serve as critical baseline data for informed control and prevention of the disease in the country. Overall, we recommend the need for all-inclusive brucellosis control programmes, taking into consideration the roles of meat handlers and traditional lifestyles in the epidemiology of human brucellosis in Nigeria. Such risk factors might not be limited to Nigeria alone, but also common among other developing countries particularly in sub-Saharan Africa. As such, there is a need for both national and international relevant stakeholders to synergistically formulate policies towards raising awareness campaigns about zoonoses in general among the high risk occupational groups in developing nations of the world.

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The authors declare that there is no conflict of interest whatsoever.

Authors' contributions

HKA initiated the concept and design of the study and wrote the manuscript; PIA did the statistical analysis and was involved in the writing of the manuscript; and MAO did the data collection and wrote the first draft of the manuscript. All authors read and approved the final version of the manuscript.

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ORIGINAL ARTICLE

Factors associated with regular sunscreen use by medical students of a Peruvian university

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Keywords

Sun protection • Sunscreen • Ordinal logistic regression

Summary

Introduction. *Use of sunscreen is encouraged to reduce the risk of skin pathologies caused by radiation. It is important to acknowledge the associated factors that promote or hinder sunscreen use in young populations as to design better prevention policies.*
Objective. *To determine the factors associated with regular sunscreen use among first year medical students from a Peruvian university.*
Materials and methods. *A cross-sectional study was performed. Our population was first-year medical students from a Peruvian university. We administrated an electronic survey to evaluate socio-demographic data, as well as student knowledge, attitudes, and practices regarding photo-protection. We used ordinal logistic regression to analyze the factors associated with sunscreen use.*
Results. *Of 420 first-year students, 299 completed our survey. We found that 53.5% of the participants were less than 18 years old, 63.2% were female, 9.3% (females more than males) responded that a sunburn was worth it to look tan, and 38.1% always or almost always used sunscreen during the summer. Factors associated with sunscreen use in the ordered logistic adjusted regression were male sex (OR = 0.50, IC95% = 0.34-0.86), participation in photo-protection workshops within the last year (OR = 2.40, IC95% = 1.28-4.37), and having somebody to remind them the use of sunscreen during the last three months (OR = 3.80, IC95% = 1.28-11.20).*
Conclusions. *In our sample, a higher sunscreen use was more often observed among female participants, those who attended skin protection workshops, and those reminded to use sunscreen. This highlights the importance of educational and reminder activities in the adoption of protective habits, such as sunscreen use.*

Introduction

Regulated sun exposure is beneficial to human beings because it prevents autoimmune diseases, helps produce vitamin D3, is beneficial for certain skin diseases such as psoriasis, and increases the serum levels of endorphins [1]. However, excessive sun exposure is related to the development of skin cancer, skin damage, photoaging, eye problems, DNA mutations, and immune system damage [2].
Skin cancer is the major consequence of excessive sun exposure [3, 4]. According to the World Health Organization (WHO), one of every three cancers detected in the world is a skin cancer [5]. As such, WHO recommends many photo-protective methods such as: seeking shade, use of sunscreen, skin-covering clothes, hats, and sunglasses with UV filters [6].
Up to 80% of the radiation absorbed during one's entire life is absorbed during childhood and adolescence [7, 8]. So, the best way to prevent the consequences of excessive sun exposure, including skin cancer, is to promote regular use of photoprotective methods from early ages [3, 4].
Use of sunscreen is one of the most recommended photoprotective methods. Unfortunately, it is frequently sub-optimally utilized, especially among adolescents and young adults [9]. Use of sunscreen in teenagers and young adults has been evaluated in several studies where the prevalence fluctuates between 26% and 78% [10-12] It has been found that sunscreen use is related to some characteristics such as female sex, adult supervision, habits ingrained during childhood, prior awareness, previous sunburns, expertise in the topic, previous use of tanning beds, warmer climates, and skin color [12-16]. However, these factors are context-dependent, so they can vary between regions.
Little has been described about these factors in Latin America [14, 17]. This lack of information hinders the design and enhancement of public policies aimed to promote the use of photoprotective methods in young people, in order to prevent a variety of skin diseases, including skin cancer. Thus, the aim of this study is to assess the factors associated with the regular use of sunscreen in university medical students.

Materials and methods

Study design and setting

During April 2014, we conducted a descriptive study among first year medical students from the Universidad de San Martín de Porres (USMP). The USMP is a private university located in Lima, the capital city of Peru.

Students are usually middle class and come from all over the country.

Participants

Participants were all first year medical students who were enrolled according to a USMP database. By 2014, a total of 420 students were registered at the university. We perform the survey in those who agreed to participate in the study after reading an informed consent. Participants whose surveys were less than 80% complete were excluded from the analyses.

Procedure

Prior to the completion of this study, we obtained proper approval from the USMP ethics committee (IRB). We developed a consent form and survey based on the current literature. Both formats were posted on the USMP "virtual classroom", so all first-year students could access both documents with a personalized password.
We approached first-year students during class periods in the computer lab to request their participation. The researchers were present during the survey completion to answer participants' questions. Students unable to take the survey during their class period were granted the opportunity to complete it outside class hours.

Variables

Use of sunscreen

The use of sunscreen was measured by the statement: "during the last summer, when you were out in the sun, you used sunscreen…", and the options: "never, almost never, sometimes, almost always, or always". Later, this variable was categorized into three categories (Never/Almost never, Sometimes, and Always/Almost always) to perform the ordinal logistic regression.
For the record, Peruvian summer occurs during January-March, and the survey was completed during April.

Other variables

The survey included five sections: demographic data (sex, age, place of birth, diagnosed skin disease, familiar or known person with skin cancer), self-identified skin phototype according to the Fitzpatrick classification (18), attendance to a photoprotective workshop, having someone remind you to use sunscreen in the last three months, knowledge of sun protection, attitudes regarding sun exposure, and assessment of usual photo-protective methods (use of sunglasses with UV filters, hats or caps, umbrellas and long sleeves).

Statistical analysis

Data from the surveys were extracted from the virtual classroom, and exported into a Microsoft Excel database. Subsequently, surveys with less than 80% completion were eliminated from the database.
Data was analyzed using STATA v13 (StataCorp, College Station, TX, US). For descriptive analysis, we used frequencies and percentages. For bivariate analysis, we used Chi-squared tests with level of significance of 5% or Fisher's exact test when expected frequencies in contingency tables were less than five.
Finally, as the outcome variable (sunscreen use) had an ordinal level of measurement, we used crude and adjusted ordinal logistic regression after testing the proportional odds assumption to determine the associated factors. Adjusted regression included all variables tested in the crude analysis.

Ethical issues

Participation in this study was voluntary, as stated in the consent form. To ensure the anonymity of the participants, personal data (such as names, numbers of identity documents, and so on) were not requested. Moreover, the database was handled only by the researchers.

Results

Participants characteristics

We requested the participation of all 420 first-year medical students enrolled in the USMP in 2014, from which 321 (76.4%) took the survey. After quality control, 22 surveys were eliminated for being incomplete; leaving 299 (71.2%) surveys for analysis.
Univariate analysis reveals that 53.5% of the participants were less than 18 years old, 63.2% were female, and 67.2% were born in Lima (capital city of Peru). With respect to the skin phototype, 46.1% had phototypes I, II or III, and 40.5% had phototype IV. With respect to the personal and familiar history, 15.7% had a skin disease, and 8.0% had a family member with known skin cancer (Tab. I).

Knowledge, attitudes and practices

Around 97.0% of the participants correctly answered that solar radiation is a major cause of skin cancer, but only 72.9% correctly answered that a sunscreen of SPF 15 is not better than one of SPF 30, moreover, only 23.1% correctly answered that on a cloudy day it is also necessary to use the sunscreen (Tab. II).
Regarding perceptions, 87.0% of the participants affirm that it is worth to use sunscreen to avoid future health problems, 18.2% believe that tan people look more attractive, and 9.3% responded that it is worth to get a sunburn to look tan. This last perception was higher among females than males ($p = 0.021$).
With respect to the use of photoprotective methods, we found that the respondents always or almost always walked in the shade (66.9%), used sunscreen (38.1%), and wore long pants (30.1%). The use of sunscreen and long pants were higher among females than males ($p = 0.010$ and $p = 0.011$, respectively) (Tab. III).

Factors associated with the use of sun protection

Factors directly associated with a higher use of sunscreen in the ordered logistic adjusted regression

Tab. I. Demographic data in first-year medical students at a private university in Lima, Peru 2014 (N = 299).

Characteristics	N (%)
Age	
< 18 years old	160 (53.5)
18-19	112 (37,5)
20 or more	27 (9,0)
Sex	
Female	189 (63.2)
Male	110 (36.8)
Place of birth	
Peru: Lima City	201 (67.2)
Peru: Other	81 (27.1)
Foreign	17 (5.7)
Fitzpatrick Skin phototype	
I-III	138 (46.1)
IV	121 (40.5)
V- VI	40 (13.4)
Diagnosed skin disease	
No	252 (84.3)
Yes	47 (15.7)
Familiar or known with skin cancer	
No	275 (92.0)
Yes	24 (8.0)
Have you ever attended to a workshop about photoprotective methods?	
Never	181 (60.5)
Yes / Long ago	66 (22.1)
Yes / This year	52 (17.4)
Did somebody remind you to use sunscreen in the last three months?	
Never	4 (1.3)
During childhood	11 (3.7)
During the last months	11 (3.7)
Both	273 (91.3)

Tab. II. Knowledge about solar exposure and the use of sunscreen in first-year medical students at a private university in Lima, Peru 2014.

Knowledge	Yes N (%)	Do not know N (%)	No N (%)
Solar radiation is a major cause of skin cancer?	290 (97.0)	6 (2.0)	3 (1.0)
A person with dark skin also needs to use sunscreen?	289 (96.7)	4 (1.3)	6 (2.0)
The use of sunscreen prevents skin cancer?	276 (92.3)	5 (1.7)	18 (6.0)
A sunscreen of SPF 15 is better than one of SPF 30?	22 (7.4)	59 (19.7)	218 (72.9)
On a cloudy day it is also necessary to use the sunscreen?	69 (23.1)	17 (5.7)	213 (71.2)
When using sunscreen, Can you expose to the sun without risk?	70 (23.4)	11 (3.7)	218 (72.9)

were: participation in at least one workshop about photoprotective methods in the last year (OR = 2.37, IC95% = 1.28-4.37) and having somebody to remind them the use of sunscreen during the last summer (OR = 3.78, IC95% = 1.28-11.21). While male sex (OR = 0.54, IC95% = 0.34-0.86) was inversely associated (Tab. IV).

Tab. III. Perceptions and practices about solar exposure and photoprotective methods in first-year medical students at a private university in Lima, Peru 2014.

	Total N = 299	Male N = 110	Female N = 189	p*
Perceptions (Agree with)				
It is worth to use sunscreen to avoid future health problems	260 (87.0)	96 (87.3)	164 (86.8)	0.526
Tan people is more attractive	54 (18.2)	24 (21.8)	30 (15.9)	0.129
It is worth it to get a sunburn to look tan	28 (9.3)	5 (4.6)	23 (12.2)	0.021
Tan people is more healthy	20 (6.7)	4 (3.6)	16 (8.5)	0.082
Practices during the last summer (Always/Almost always)				
Walk in the shadow	200 (66.9)	73 (66.4)	127 (67.2)	0.491
Sunscreen	114 (38.1)	32 (29.1)	82 (43.4)	0.010
Large pants	90 (30.1)	24 (21.8)	66 (34.9)	0.011
Sunglasses with UV filters	86 (28.8)	25 (22.7)	61 (32.3)	0.051
Not going out in the hours of higher radiation	86 (28.8)	28 (25.5)	58 (30.7)	0.203
Hats or caps	53 (17.7)	25 (22.7)	28 (14.8)	0.059
Umbrella	37 (12.4)	11 (10.0)	26 (13.8)	0.223
Long sleeves	24 (8.0)	11(10.0)	13 (6.9)	0.228

* Fisher's exact test

Tab. IV. Factors associated with the use of sunscreen in first-year medical students at a private university in Lima, Peru 2014.

Characteristics	Use of sun screen N (%)			Crude model			Adjusted model*		
	Never/ Almost never	Sometimes	Always/ Almost always	OR	IC 95%	p	OR	IC 95%	p
Age									
< 18 years	31 (19.4)	70 (43.7)	59 (36.9)	Ref.			Ref.		
≥ 18 years	37 (26.6)	47 (33.8)	55 (39.6)	0.92	(0.60-1.40)	0.700	0.82	(0.53-1.27)	0.383
Sex									
Female	33 (17.5)	74 (39.1)	82 (43.4)	Ref.			Ref.		
Male	35 (31.8)	43 (39.1)	32 (29.1)	0.50	(0.32-0.78)	0.002	0.54	(0.34-0.86)	0.009
Fitzpatrick Skin Phototype									
I-III	24 (17.4)	53 (38.4)	61 (44.2)	Ref.			Ref.		
IV	32 (26.4)	49 (40.5)	40 (33.1)	0.61	(0.39-0.96)	0.035	0.67	(0.41-1.09)	0.103
V-VI	12 (30.0)	15 (37.5)	13 (32.5)	0.56	(0.29-1.07)	0.080	0.58	(0.30-1.14)	0.115
Diagnosed skin disease									
No	59 (23.4)	95 (37.7)	98 (38.9)	Ref.			Ref.		
Si	9 (19.2)	22 (46.8)	16 (36.0)	0.96	(0.55 - 1.70)	0.899	1.00	(0.55-1.78)	0.971
Familiar or known with skin cancer									
No	60 (21.8)	110 (40.0)	105 (38.2)	Ref.			Ref.		
Si	8 (33.3)	7 (29.2)	9 (37.5)	0.75	(0.34 - 1.68)	0.489	0.8	(0.34-1.71)	0.509
Have you ever attended to a workshop about photoprotective methods?									
Never	47 (25.9)	70 (38.7)	64 (35.4)	Ref.			Ref.		
Yes/ Long ago	14 (21.2)	31 (47.0)	21 (31.8)	1.01	(0.60 - 1.70)	0.954	1.00	(0.59 - 1.71)	0.983
Yes/ In the last year	7 (13.5)	16 (30.8)	29 (55.7)	2.34	(1.29 - 4.27)	0.005	2.37	(1.28 - 4.37)	0.006
Did somebody remind you to use sunscreen during the last summer?									
No	9 (60.0)	3 (20.0)	3 (20.0)	Ref.			Ref.		
Yes	59 (20.8)	114 (40.1)	111 (39.1)	1.56	(0.49 - 2.62)	0.004	3.78	(1.28 - 11.21)	0.016

*Adjusted model include every variable presented

Discussion

Knowledge

In this study, we found that 97.0% of the participants knew about the relationship between sun exposure and skin cancer. These results are consistent with other studies completed in Australia where 80% of teenagers were competent regarding the dangers of sun exposure [16], and with research made in United States of America (USA), where 89% of the adolescents knew about the association between unprotected sun exposure and skin cancer [19].

Fewer participants answered correctly about adequate sunscreen use: 71.2% answered that on a cloudy day it is not necessary to use the sunscreen, and 7.4% answered that a sunscreen of SPF 15 is better than one of SPF 30. These percentages are similar to other studies [19], and reflect that information regarding correct sunscreen use is not yet widely dispersed. These results suggest the necessity to improve population-level knowledge on this subject, as previous studies have shown the positive association between high knowledge and a lower sunburn incidence [13].

Practices and perceptions

The perception that it is worth to get a sunburn to look tan was higher among females than males. Results were similar with other studies [13, 16]. This may be due to the arraigned social perceptions of beauty and fashion, which are especially strong in female adolescents [20, 21]. Educational campaigns must take this into consideration and make appropriate recommendations [22].

The most commonly used photoprotective methods were walking in the shade, use of sunscreen, and the use of long pants. Nevertheless, sunscreen was used "always" or "almost always" by only 38.1% of the population. These results are consistent with previous data, where only 31.4% of the adolescents used sunscreen frequently [19].

Nearly a third of the participants used sunglasses with UV filters "always" or "almost always". Similar results found in other studies was the use of sunglasses (32.2%) as one of the most common sun-protection behaviors [19]. Although not statistically significant ($p = 0.051$), our findings suggest that women are more likely to use sunglasses (32.3%) than with men (22.7%). These results were consistent with previous studies, where men use sunglasses less often [16]. This could reflect a difference in sun protection awareness, or in fashion customs, between males and females.

Factors associated with the use of sunscreen

In the multiple regression analysis, a higher use of sunscreen was associated with female sex. This result is similar to other studies [13, 16], and could be explained by differences in cultural roles between young males and females. Women are more concerned with personal care and skin rotection than men [23].

Attending a sunscreen workshop in the last year was also associated with increased sunscreen use. Other studies found that adequate educational campaigns could eradicate myths and improve the quality of sunscreen use among young people [15, 17].

Other protective factor was having a person who has reminded them to use to use sunscreen in the last three months. In general, it seems that one of the biggest barriers hindering sunscreen use is forgetting to apply it [24] and lack of habit [14].

Recommendations

Our findings suggest that it is necessary to organize sunscreen educational activities for middle class urban children and adolescents. These activities should include information concerning the correct sunscreen use, and sex-specific recommendations, such as avoid tanning for women and use of photoprotective methods for men. These activities could be implemented in schools, universities, and recreational settings [25].

Although not all young people have somebody to remind them about this topic, there are new methods to inform youth about sun protection (i.e., text messages or Smartphone applications) , which have already been used effectively [24]. These methods should be tested and implemented in young Peruvian population.

Nevertheless, the participants of this study are urban middle-class medical students. Therefore, our findings cannot be extrapolated to populations of lower socioeconomic status or rural dwellers who probably have different challenges in accessing educational and reminder activities that help to reinforce healthy habits.

Limitations

The present study has some limitations: first, it is possible that participants, well-educated medical students, have a greater interest and knowledge-fund than the general Peruvian population. Moreover, the use of sunscreen as well as other variables were collected through an electronic survey, which are subjected to recall bias, as participants surveyed are in their first years of study, so we believe that their knowledge is not very different from that of other university freshmen.

Conclusions

In conclusion, our findings show that the main factors related to a higher sunscreen use in our population are being female, having attended safe-sun workshops, and having a person who reminds them to use sunscreen. Moreover, perceptions and practices related solar exposure and photoprotective methods differ according to sex. These results should be taken into consideration when developing educational programs aimed at young middle class urban populations.

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Authors' contribution

PR-G and AT-R contributed to the conception and design of this study. PR-G planned and performed data collection and prepared the first manuscript draft. AT-R and MGM-P provided feedback for all the manuscript versions. MGM-P and AT-R performed the statistical analyses. All authors revised and approved the final version of the manuscript.

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ORIGINAL ARTICLE

Using the cytokinesis-block micronucleus cytome assay to evaluate chromosomal DNA damage in chronic renal patients undergoing bicarbonate haemodialysis and haemodiafiltration

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Keywords

Chronic Renal Failure (CRF) • DNA damage • Micronucleus (MN)

Summary

Introduction. *Chronic Renal Failure (CRF) patients are considered to show genomic instability and are associated with a high risk of both cardiovascular diseases and cancer. We explored DNA damage due to two dialysis treatments in 20 patients undergoing bicarbonate haemodialysis (BD), 20 undergoing haemodiafiltration (HDF) and 40 healthy subjects.*

Methods. *The cytokinesis-block micronucleus (MN) assay was performed on peripheral blood lymphocytes to evaluate genetic damage.*

Results. *A higher frequency of MN in the dialysis groups compared with controls was found. The results do not show a relationship between genetic instability and the type, frequency and duration of haemodialysis. The average BD and HDF treatment time was respectively 3.8 ± 6.3 and 3.7 ± 3.9 yrs. CAT and scintigraphy was independently correlated with high levels of MN.*

Conclusions. *Overall, the frequency of MN in CRF patients undergoing dialysis therapy was observed to be higher. Further studies need to be performed on a larger number of patients and for a longer period.*

Introduction

Chronic Renal Failure (CRF) is a progressive disease with loss of kidney function over time [1]. The early stages of CRF (stages 2 and 3) are characterized by a decrease in the glomerular filtration rate (the best parameter for categorising kidney function) and are generally asymptomatic. Advanced stages of the disease (4 and 5) are manifested by a severely decreased glomerular filtration rate accompanied by clinical complications (hypertension, anaemia, bone disease), requiring renal replacement therapy when end-stage renal disease is reached [2].

CRF patients, regardless of whether they are receiving dialysis, present a high risk of cardiovascular pathologies and cancer (mainly cervical, bladder, thyroid, and renal cell carcinoma) [3-5], as well as elevated levels of genetic damage [6, 7]. This extensive damage may be related to impairment of DNA repair. DNA lesions may induce mutations in tumour-suppressors and oncogenes that may lead to malignancies if mutagenicity is not mitigated by repair mechanisms [8].

Uraemia, microinflammation and oxidative stress [free radicals, reactive oxygen species (ROS), etc] are the main mechanisms underlying this phenomenon [6]. Indeed, evidence indicates that end-stage renal disease is associated with oxidative stress, as a result of both increased production of oxidants and weaker antioxidant defences [9-11].

This situation is aggravated by a series of events induced by dialysis treatment. Continuous contact of peripheral blood with dialysis membranes promotes the activation of leukocytes that produce various inflammatory mediators (e.g. complement and platelet-activating factor) [12].

Renal Replacement Therapies (RRT) involve peritoneal (or intracorporeal) dialysis, which is a blood-filtering method that uses the peritoneum, the serous membrane that lines the abdominal wall, to allow exchanges between blood and dialysis fluid, and extracorporeal dialysis or haemodialysis, in which blood circulates outside the body, using an artificial membrane in an external filter to remove waste products [13].

The types of dialysis treatment respond to different therapeutic needs, specifically the type and size of toxic molecules to be removed. Diffusive and diffusive-convective techniques are both currently used [14]. The former include Acetate and Bicarbonate Dialysis (BD), while the latter include Haemodiafiltration (HDF), an innovative diffusive-convective blood purification treatment devel-

oped from BD, consisting of a combination of Haemofiltration (HF) and conventional Haemodialysis (HD) [15]. HDF combines the advantages of the diffusive method of removing low molecular weight solutes with those of convective treatment, which removes substances with medium/high molecular weight [16, 17].
Several studies have found high levels of genetic damage in patients with CRF suffering from uraemia and oxidative stress, detected by methods such as sister-chromatid exchange, the comet test and micronucleus assays [8, 18, 19]. Indeed, both CRF and the long-term HD therapy used to treat it can cause genomic damage, leading to single and double-strand breaks, alkali-labile sites and formation of micronuclei (MN), in addition to reduction of DNA repair capacity [18, 20]. MN are DNA-containing particles that occur during mitosis and result from unrepaired DNA double-strand breaks, leading to chromatin fragments or whole chromosomes being distributed incorrectly. MN frequency is considered a good surrogate biomarker for detecting genetic damage and evaluating cancer risk [21, 22]. The MN assay is performed on human lymphocytes because they are excellent markers of exposure; they circulate for years or even decades through different organs and accumulate DNA damage during their lifespan [23-25].
The aim of the present study is to evaluate DNA damage in CRF patients undergoing BD and HDF dialysis techniques compared with a control group, by evaluating MN frequency in peripheral blood lymphocytes (PBL).

Methods

Subjects. The study was carried out on a total of 80 individuals including 40 CRF patients (20 undergoing BD and 20 undergoing HDF) and 40 healthy controls.
Patients aged less than 18 years, pregnant, with malignancies, with bacterial or viral infections, hepatic impairment, or undergoing treatment with anti-inflammatory agents, cytostatics or immunosuppressive drugs were excluded. Healthy volunteers who did not meet the exclusion criteria served as control subjects.
All participants in the study were recruited at the "*I. Veris Delli Ponti*" hospital in Scorrano from May 2013 to December 2014 and completed a questionnaire requesting general details and information on smoking habits, alcohol intake, occupational exposure and risk factors for cancer.
This study was approved by the local institutional Ethics Committee and informed consent was obtained from each patient enrolled.
Lymphocyte Culture and Cytokinesis-Block Micronucleus (CBMN) Cytome Assay**.** Blood samples were obtained for each subject by venipuncture using heparinized vacutainers and sent directly to the Laboratory of Hygiene of University of Salento.
300 µl of blood sample was added to 4.7 ml of Karyotyping medium. At 44-h incubation, 100 µl of cytochalasin B was added to the culture to arrest cytokinesis.
After 28-h incubation, the cultures were harvested by centrifugation at 2000 rpm for 4 min at 25°C and treated with a hypotonic solution (112 mg KCl/20 ml of deionized water) for 10 min. The supernatant was discarded after each centrifugation, leaving approximately 0.5 ml of suspension. 0.4 ml of acetic acid/methanol (5:3) solution was added to the culture 10 min later. The cells were centrifuged again and 5 ml of methanol was added. After a further centrifugation, the cell suspension was twice fixed in a methanol/acetic acid solution (7:1) and then centrifuged again. The tubes were then placed in a freezer for two hours. The pellet was resuspended and 3 drops were placed on a clean slide kept at -20°C. The slides were stained with Giemsa solution. Afterwards, they were washed with distilled water and left to dry overnight.
For each sample, 1000 binucleated cells were scored under optical microscope for MN analysis, following the criteria for determining MN [26]. We evaluated MN frequency as the number of micronucleated cells per 1000 cells (‰). To avoid differences between observers, the same individual carried out the microscopic analyses.
The Nuclear Division Index (NDI), a cell proliferation index, was calculated by scoring mono-, bi-, tri- and tetranucleated cells in accordance with Eastmond and Tucker [27].
Statistical analysis. All analyses were performed using SPSS 18.0 (Chicago, USA). Continuous variables were expressed as mean ± standard deviation (SD), whereas categorical variables were expressed in absolute and percentage values.
For continuous variables, differences between groups were compared by the Mann-Whitney test and 1-way Analysis of Variance (ANOVA), where applicable. Homogeneity of variance was evaluated using the Levene test. ANOVA was performed with a Brown-Forsythe adjustment for heteroscedasticity, and with a post-hoc Tukey test or Dunnett's T3 procedure for multiple comparisons of unequal variances in order to determine which groups differ from the others.
Pearson's chi-square and the likelihood ratio chi-square were used for proportions.
Univariate and multivariate logistic regression analyses were performed to examine predictors of abnormal MN frequency. Variables that proved to be associated with higher MN frequency ($p < 0.25$) in univariate analyses were inserted in a multivariate logistic regression model in order to investigate independent predictors of high frequency. Stepwise regression analysis was performed in order to select the variables adopted in the multivariate model. For all analyses, a p-value of < 0.05 was considered to be statistically significant.

Results

The demographic characteristics and risk factors of CRF patients and healthy controls are shown in Table I. The average age of the control group was lower (53.2 ± 10.2) than that of patients treated by BD (57.0 ± 12.0) and HDF (59.8 ± 10.1), although the differences were not statistically significant. The differences between patients

Tab. I. Characteristics of patients with bicarbonate hemodialysis (Group 1), hemodiafiltration (Group 2), and control group.

	Group 1 (n = 20)	Group 2 (n = 20)	p-value	Control Group (n = 40)	p-value
Age (± SD)	57.0 ± 12.0	59.8 ± 10.1	0.685*	53.2 ± 10.2	0.075**
Gender, male, n (%)	12 (60.0)	13 (65.0)	0.774^	27 (64.7)	0.623^^
Risk factors					
Diagnostic test					
Radiography, n (%)	18 (90.0)	16 (80.0)	0.661^	38 (95.0)	0.074
CAT, n (%)	12 (60.0)	11 (55.0)	0.927^	7 (17.5)	0.749^^
Scintigraphy, n (%)	17 (85.0)	13 (65.0)	0.273^	1 (2.9)	0.000^^
Angiography, n (%)	4 (20.0)	5 (25.0)	0.519^	0 (-)	0.001^^
Mammography, n (%)	8 (40.0)	6 (30,0)	0.921^	3 (8.8)	0.006^^
Radiotherapy, n (%)	0 (-)	0 (-)	-	0 (-)	-
MRI, n (%)	0 (-)	1 (5,0)	1.000^	8 (20.0)	0.235^^
Echography, n (%)	20 (100)	20 (100)	1.000^	18 (45.0)	1.000^^
Smoke, n (%)	13 (65.0)	6 (30,0)	0.057^	14 (35.0)	0.025^^
Years of smoking (± SD)	17.9 ± 7.2	15.5 ± 6.3	0.848*	16.3 ± 10.7	0.829**
Alcohol (all), n (%)					
Wine	15 (78.9)	15 (78.9)	0.715^	12 (30.0)	0.000^^
Beer	16 (84.2)	14 (73.7)	0.715^	12 (30.0)	0.000^^
Spirits	6 (31.6)	2 (10.5)	0.236^	7 (17.5)	0.262^^
Diabetes, n (%)	3 (15.0)	6 (30.0)	0.449^	0 (-)	0.000^^
Hypertension, n (%)	15 (75.0)	17 (85.0)	0.693^	5 (12.5)	0.000^^
Intercontinental travel, n (%)	1 (5.0)	1 (6,70)	0.468^	2 (5.0)	1.000^^
Mobile phone repeaters, n (%)	0 (-)	0 (-)	-	6 (15.0)	0.012^^
Residential area					
Town centre, n (%)	13 (65.0)	13 (65.0)	0.497^	16 (41.2)	0.001^^
Suburban, n (%)	3 (15.0)	1 (5.0)		20 (50.0)	
Rural area, n (%)	4 (20.0)	6 (30.0)		4 (8.8)	
Plan home					
Ground floor, n (%)	14 (70.0)	16 (80.0)	0.344^	18 (44.1)	0.016^^
First floor, n (%)	2 (10.0)	3 (15.0)		17 (42.5)	
Second floor n (%)	4 (20.0)	1 (5.0)		5 (11.8)	
Education level					
Primary school, n (%)	9 (45.0)	6 (30.0)	0.290^	1 (2.5)	0.000^^
Secondary school, n (%)	8 (4.0)	16 (30.0)		14 (35.0)	
High school diploma, n (%)	2 (10.0)	6 (30.0)		15 (37.5)	
Degree, n (%)	1 (5.0)	2 (10.0)		10 (25.0)	
Professional exposure					
Ionizing radiation, n (%)	0 (-)	0 (-)	-	0 (-)	-
Pesticides, n (%)	0 (-)	0 (-)	-	0 (-)	-
Chemicals, n (%)	0 (-)	0 (-)	-	6 (11.8)	0.012^^
Heavy metals, n (%)	0 (-)	0 (-)	-	0 (-)	-
Anesthetic gases, n (%)	7 (35.0)	7 (35.0)	0.740^	1 (2.9)	0.056^^
Surgery, n (%)	7 (35.0)	8 (40.0)	1.000^	13 (32.5)	0.849^^
Kidney transplant, n (%)	4 (20.0)	1 (5.0)	0.442^	0 (-)	-
Time hemodialysis					
≤ 5 years, n (%)	16 (80.0)	16 (80.0)	0,675^	0 (-)	-
> 5 years, n (%)	4 (20.0)	4 (20.0)		0 (-)	
Frequency hemodialysis					
3 time a week	1 (5.0)	7 (35.0)	0.048^	0 (-)	-
> 3 time a week	19 (95.0)	13 (65.0)		0 (-)	
Kidney failure					
Glomerulonephritis, n (%)	8 (40.0)	5 (25.0)	0.399^	0 (-)	-
Nephroangiosclerosis, n (%)	5 (25.0)	5 (25.0)		0 (-)	
Diabetic nephropathy, n (%)	3 (15.0)	7 (35.0)		0 (-)	
Urethral reflux, n (%)	0 (-)	2 (10.0)		0 (-)	
Polycystic kidney, n (%)	1 (5.0)	1 (5.0)		0 (-)	

	Group 1 (n = 20)	Group 2 (n = 20)	p-value	Control Group (n = 40)	p-value
ANCA vasculitis, n (%)	1 (5.0)	0 (-)		0 (-)	
Malformation uropathy, n (%)	1 (5.0)	0 (-)		0 (-)	
Chronic rejection, n (%)	1 (5.0)	0 (-)		0 (-)	

Legend: SD, Standard Deviation; CAT, Computed Axial Tomography, MRI, Magnetic Resonance Imaging.
* HSD di Tukey
** ANOVA
^ Pearson's χ^2 test
^^ Likelihood ratio chi-square

Tab. II. Cytogenetic parameters in the studied populations.

	Group 1			Group 2			Control Group			
	N	Mean ± SD	(median)	N	Mean ± SD	(median)	N	Mean ± SD	(median)	p-value
MN/1,000										
Men	12	14.25 ± 9.77	(13.50)	13	13.77 ± 6.76	(14.00)	28	5.88 ± 2.86	(5.00)	0.002*
Women	8	13.63 ± 5.15	(15.50)	7	23.86 ± 9.25	(23.00)	12	7.67 ± 1.97	(8.00)	0.009*
Total	20	14.0 ± 8.07	(14.50)	20	17.30 ± 8.96	(15.50)	40	5.88 ± 2.86	(6.00)	0.001*
Time of hemodialysis										
≤ 5 years	16	14.2 ± 8.83	(14.50)	16	18.2 ± 9.52	(18.00)	-	-	-	0.775^
> 5 years	4	13.2 ± 4.65	(14.00)	4	13.7 ± 5.80	(14.50)	-	-	-	0.725^
		p = 0.841^			p = 0.390^					
Frequency of hemodialysis										
≤ 3 time a week	19	13.8 ± 8.24	(14.00)	13	18.4 ± 6.33	(20.00)	-	-	-	0.355
> 3 time a week	1	18.0	(-)	7	15.3 ± 12.91	(9.00)	-	-	-	-
		p = -			p = 0.567^					
NDI										
Men	12	5.69 ± 4.71	(5.61)	13	4.25 ± 2.98	(4.01)	28	1.14 ± 1.18	(0.58)	0.003*
Women	8	2.65 ± 2.82	(2.10)	7	4.71 ± 4.48	(3.14)	12	1.39 ± 1.92	(0.57)	0.258*
Total	20	4.47 ± 4.26	(3.08)	20	4.41 ± 3.47	(3.58)	40	0.94 ± 1.31	(0.58)	0.001*

Legend: SD, Standard Deviation; MN, micronucleus; NDI, Nuclear Division Index.
*ANOVA was performed with a Brown-Forsythe adjustment for heteroscedasticity and with Dunnett's T3 procedure for multiple comparisons of unequal variances.
^ Test U di Mann-Whitney.

Tab. III. Univariate and multivariate logistic regression analysis demonstrating the relationship of micronucleus (MN) frequency with most important experimental variables in dialysis patients.

	Univariate		Multivariate	
	OR (95% CI)	p	OR (95% CI)	p
Age (± SD)	1.14 (0.51-2.58)	0.742	-	
Gender, male, n (%)	2.43 (0.65-9.07)	0.183	2.19 (0.14-34.90)	0.577
Risk factors				
Diagnostic test				
- Radiography, n (%)	1.40 (0.22-8.72)	0.715	-	
- CAT, n (%)	2.20 (0.58-8.28)	0.236	7.31 (0.90-59.30)	0.062
- Scintigraphy, n (%)	0.33 (0.08-1.46)	0.139	0.09 (0.01-1.01)	0.051
- Angiography, n (%)	1.27 (0.28-5.68)	0.758	-	
- Mammography, n (%)	1.89 (0.50-7.09)	0.345	-	
Smoke, n (%)	0.51 (0.14-1.85)	0.299	-	
Alcohol				
- Wine	0.33 (0.08-1.46)	0.139	12.10 (0.00-0.00)	0.997
- Beer, n (%)	0.18 (0.04-0.88)	0.026	0.00 (0.00-0.00)	0.996
- Spirits, n (%)	0.43 (0.07-2.46)	0.321	-	
Diabetes, n (%)	3.00 (0.69-13.12)	0.139	4.10 (0.38-44.79)	0.247
Hypertension, n (%)	1.84 (0.31-10.92)	0.489	-	

	Univariate		Multivariate	
Intercontinental travel, n (%)	1.53 (0.09-26.43)	0.769	-	
Residential area				
- Town centre, n (%)	0.83 (0.22-3.12)	0.787	-	
- Suburban, n (%)	5.31 (0.50-56.39)	0.134	10.06 (0.27-377.53)	0.212
- Rural area, n (%)	0.56 (0.12-2.60)	0.450	-	
Plan home				
- Ground floor, n (%)	9.00 (1.01-80.13)	0.016	4.63 (0.14-155.21)	0.392
- First floor, n (%)	0.00 (0.00-0.00)	0.018	0.00 (0.00-0.00)	0.995
- Second floor n (%)	0.33 (0.03-3.30)	0.309	-	
Education level				
- Primary school, n (%)	1.56 (0.42-5.72)	0.506	-	
- Secondary school, n (%)	0.47 (0.12-1.88)	0.273	-	
- High school diploma, n (%)	0.88 (0.18-4.32)	0.871	-	
- Degree, n (%)	3.29 (0.27-39.66)	0.332	-	
Professional exposure				
- Anesthetic gases, n (%)	0.76 (0.20-2.90)	0.684	-	
- Surgery, n (%)	0.64 (0.17-2.41)	0.502	-	
Kidney transplant, n (%)	0.33 (0.03-3.30)	0.309	-	
Type of hemodialysis, n (%)	1.52 (0.42-5.43)	0.518		
Time hemodialysis	0.43 (0.07-2.46)	0.321	-	
Frequency hemodialysis	0.88 (0.18-4.32)	0.871	-	
Kidney failure				
- Glomerulonephritis, n (%)	0.56 (0.14-2.26)	0.404	-	
- Nephroangiosclerosis, n (%)	1.73 (0.41-7.33)	0.459	-	
- Diabetic nephropathy, n (%)	3.00 (0.69-13.12)	0.139	4.10 (0.37-44.79)	0.247
- Urethral reflux, n (%)	1.53 (0.09-26.43)	0.769	-	

Legend: OR, Odds Ratio; SD, Standard Deviation; CAT, Computed Axial Tomography, MRI, Magnetic Resonance Imaging.
Variables showing a tendency of association with abnormal MN frequency ($p < 0.25$) in the univariate analysis were included in the multivariate model.

on dialysis and controls are linked to the difficulty of recruiting healthy individuals of the same age as patients.
The risk factor analysis showed no significant difference between the two groups of patients undergoing dialysis, while highly significant differences emerged among the three groups in terms of their exposure to scintigraphy ($p < 0.000$), angiography ($p < 0.001$), mammography ($p < 0.006$), mobile phone repeaters ($p < 0.012$) and chemicals ($p < 0.012$), as well as cigarette smoking ($p < 0.025$), wine and beer consumption (both $p < 0.000$), diabetes ($p < 0.000$), hypertension ($p < 0.000$), residential area ($p < 0.001$), storey of residence (i.e. ground floor, first floor, etc.) ($p < 0.016$) and level of education ($p < 0.000$).
The results of the MN assays on PBL show significantly higher frequency in the groups on dialysis than controls ($p < 0.001$), in both males ($p < 0.002$) and females ($p < 0.009$) (Tab. II). No difference was observed between BD and HDF patients and no correlation was observed between the number of MN and the duration or weekly frequency of treatment.
In addition, as a measure of cytotoxicity, NDI was found to be significantly lower in the control group ($p < 0.001$) than BD and HDF-treated patients. The frequency of MN was significantly higher in men ($p < 0.003$) than women ($p < 0.258$) (Tab. II).
Table III shows the results of the univariate and multivariate logistic regression analyses, demonstrating relationships between MN and other variables. Univariate analysis revealed that CAT, scintigraphy, wine and beer consumption, diabetes, residence in the suburbs, storey of residence, and diabetic nephropathy are significantly associated with high MN frequency. However, only CAT and scintigraphy independently correlated with high MN frequency in a multivariate logistic regression model where the variables with $p < 0.25$ in the univariate analysis were included as independent variables (Tab. III).

Discussion

Patients with Chronic Kidney Disease (CKD) have a higher risk of developing chronic degenerative diseases, such as coronary disease, strokes or transient ischemic attacks, heart failure, peripheral arterial disease, diabetes mellitus, hypertension, dyslipidemia, lung or liver disease, cancer and dementia [28]. These adverse events are associated with severe cytogenetic damage [17].
In this study, damage was assessed by CBMN assay, in patients receiving two different dialysis treatments compared with a control group of healthy subjects. CBMN is the most frequently used chromosomal biomarker for evaluating MN frequency in PBL, which is a good surrogate marker of cancer risk [26].
It is assumed that CRF patients present high levels of genetic damage, but very little is known about the ori-

gins of this damage. Patients at all stages of CRF have greater oxidative stress than healthy people but it is even more severe in patients undergoing haemodialysis [29]. The problem of oxidative stress in patients on dialysis is mainly related to the accumulation of uraemic toxins and other endogenous substances with genotoxic properties [30]. The impairment of DNA damage repair is essentially caused by increased production of ROS [31-33]. CKD (which leads to the accumulation of metabolites) and haemodialysis (which removes metabolites) are among the factors associated with DNA damage [34].

Several studies, using a variety of techniques for the detection of chromosomal damage, have shown higher levels of genetic damage in CFR patients than controls [7, 8]. This was confirmed in the current study, in which a statistical difference in MN frequency between CFR patients and healthy volunteers was observed.

The degree of chromosome damage seems to be influenced by both the stage of CKD and the dialysis technique used [19, 22], although studies show some disagreement regarding the latter. Indeed some studies show a smaller degree of DNA damage in HD than BD, while others evince the opposite [35, 36]. Our study found no significant difference in oxidative damage between patients receiving HD and BD.

Factors such as age, gender, tobacco and alcohol intake, diabetes, hypertension and level of education were not found to influence the genotoxic effect of haemodialysis treatment.

The univariate and multivariate logistic regression analyses showed that the risk factors associated with higher DNA damage are diagnostic procedures involving exposure to ionizing radiation (CAT and scintigraphy). Literature data suggest that exposure to ionizing radiation induces the formation of MN and increases the risk of cancer and cardiovascular diseases [37, 38].

Some authors have shown that DNA damage correlates with the duration of dialysis treatment after more than 7 years [18, 22].

The results of this study show no relationship between genetic instability and the type and frequency of haemodialysis. In terms of the duration of treatment, the average for the BD and HDF patients was respectively 3.8 ± 6.3 and 3.7 ± 3.9 yrs, not sufficient to assess its relationship with genetic instability.

Our results are consistent with the findings of Kan E et al., in which the average duration of dialysis treatment was approximately 3.5 years [39]. Another limitation of our study is the small sample size, which is not sufficient to distinguish between the DNA damage induced by the different treatments. Therefore, in order to expand this study, a larger number of patients, in treatment for more than 10 years, is required.

In conclusion, the results of the research provide evidence that patients undergoing dialysis show a higher frequency of nuclear anomalies, resulting in alterations of genetic material as well as failures in repair mechanisms. Both CRF and the dialysis used to treat it can contribute to chromosomal and/or genomic damage, bearing in mind that the formation of MN mainly originates from acentric chromosome fragments or whole chromosomes secluded from daughter nuclei during mitosis.

The severe DNA damage in CRF patients, exacerbated by the dialysis used to treat the condition, is relevant to the debate about possible intervention strategies to reduce the risk of cancer and cardiovascular disease. The use of highly biocompatible membranes, ultrapure dialysates and extracorporeal removal of ROS, as well as the many dietary antioxidants and pharmacological agents now being used to modulate the levels of genetic damage, need to be further investigated.

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Authors' Contributions

MG, AZ, GS and ADD conceived, designed and coordinated the research. MD'A, AI, FS and TG collected data and samples. MG, AZ, MRT and MD'A performed the data quality control. MG and FB optimized the informatics database. MG performed the statistical analyses. MG, AZ, MRT, GS, MD'A and DDA evaluated the results. MG, AZ and MRT 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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