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# Public perceptions in relation to intention to receive pandemic influenza vaccination in a random population sample: evidence from a cross-sectional telephone survey

**V Sypsa[1], T Livanios[2], M Psichogiou[3], M Malliori[4], S Tsiodras[5], I Nikolakopoulos[6], A Hatzakis (ahatzakis@med.uoa.gr)[1]**
1. Department of Hygiene, Epidemiology and Medical Statistics, Athens University Medical School, Athens, Greece
2. Opinion Marketing Research, Athens, Greece
3. First Department of Propaedeutic Medicine, Laiko General Hospital, Athens, University Medical School, Athens, Greece
4. Department of Psychiatry, Athens University Medical School, Athens, Greece
4. Department of Internal Medicine, University of Athens Medical School, Attikon University Hospital, Xaidari, Greece
5. Faculty of Political Science and Public Administration, University of Athens, Athens, Greece

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A cross-sectional telephone survey on a nationally representative sample of 1,000 Greek households was performed to assess the acceptability of the pandemic influenza A(H1N1)v vaccine, factors associated with intention to decline and stated reasons for declining vaccination. The survey was initiated the last week of August 2009 (week 35) and is still ongoing (analysis up to week 44). The percentage of participants answering they would "probably not/definitely not" accept the vaccine increased from 47.1% in week 35 to 63.1% in week 44 (test for trend: p<0.001). More than half of the people which chronic illnesses (53.3%) indicated "probably not/definitely not". Factors associated with intention to decline vaccination were female sex, age between 30-64 years, perception of low likelihood of getting infected or of low risk associated with influenza, and absence of household members suffering from chronic illnesses. For the majority of the respondents (59.8%), the main reason for intending to decline vaccination was the belief that the vaccine might not be safe. Promotion of vaccination programmes should be designed taking into account the attitudinal barriers to the pandemic vaccine.

## Introduction

One of the first priority actions following the declaration of influenza A(H1N1)v as the first pandemic of the 21st century was the timely development of a safe and effective vaccine. Although vaccination is an effective measure to reduce the number of infections, hospitalisations and deaths, modelling studies have shown that the impact of vaccination depends strongly on the time when it is initiated as well as on the coverage of the target populations [1-3]. Until the beginning of November 2009, the European Commission had granted authorisation for three specific influenza A(H1N1)v vaccines and vaccination has already started in several European countries. However, there is a major concern about the acceptability of the pandemic vaccine among target populations in several European countries. In the present study, we analysed the data from a weekly telephone survey carried out in the Greek population in order to assess the levels of acceptance of the vaccine and the related attitudinal barriers.

## Methods

### Telephone survey

A telephone survey on 1,000 households has been carried out in Greece on a weekly basis starting from the last week of August 2009 (week 35) and was still ongoing until the time of this analysis (week 44). One of the aims of the study was to assess perceptions in relation to risks of pandemic influenza A(H1N1) and the attitude towards immunisation. Proportional quota sampling was used to ensure that selected households were representative of the total of Greek households, with quotas based on household size and urban/rural location. The average household size in the selected households was 2.9 persons. The mean age of the respondents was 51.9 (standard deviation ± 17.0) years and 65.8% of them were female.

One participant per household was asked to provide answers to questions about the age and sex of the household members, knowledge and perceptions about influenza A(H1N1)v, the presence of members with chronic illnesses etc. Chronic illnesses included chronic respiratory diseases (including asthma), chronic cardiovascular diseases (except hypertension), chronic metabolic disorders (including diabetes mellitus), chronic renal and hepatic diseases, haematological disorders (including sickle cell disease), immunosuppression and chronic neurological/neuromuscular diseases. A specific question was asked concerning the willingness of the participants to accept vaccination once the pandemic vaccine becomes available: "Do you consider getting vaccinated against the novel influenza (you or the other members of your household) once the vaccine becomes available?" with five possible answers ("definitely yes", "probably yes", "probably not", "definitely not", "don't know").

### Statistical methods

The presence of trend in the intentions of the population sampled every week was evaluated using the chi-squared test for trend. The data from week 44 were further used to identify associations between questionnaire-related variables and the

reported vaccination intentions using one-way analysis of variance and the chi-squared test. A multiple logistic regression model was used to evaluate independent predictors of intention to decline the vaccine (where the answers were grouped as "definitely not/probably not" versus "definitely yes/probably yes"). A similar model was used to identify the profile of a non-negligible proportion of the sample answering "don't know" (versus "definitely yes/probably yes").

## Results

Overall, according to the most recent data of week 44, 63.1% of the sample indicated "probably not/definitely not" as their intention to get vaccinated. The trends from week 35 through week 44 in the willingness of the respondents to get the pandemic vaccine are depicted in Figure 1 (1,000 persons per week). The percentage of participants answering "definitely not" increased from 32.3% on week 35 to 45.8% in week 44 (test for trend: p<0.001). The proportion of individuals responding "definitely yes" decreased from 22.9% in week 35 to 9.1% in week 44 (test for trend: p<0.001).

Respondents' age, sex and educational attainment, the presence of chronic illness and history of seasonal influenza vaccination in the past year (of the respondents and of members of their household), presence in the household of children aged 0-12 years or of individuals aged 65 or older, and respondents' perceptions concerning the risk related to infection were associated with the reported intention towards getting vaccinated (Table 1). Women intended to decline vaccination at higher rates (67.6%) compared with men (54.4%) and were more determined in their answer (51.8% answered "definitely not"). Persons with a history of previous seasonal influenza vaccine reported intention to decline vaccination at lower rates compared with those who have not

**Figure 1**

**Trends in respondents' intention to receive pandemic vaccine, Greece, 2009 (1,000 respondents per week)**

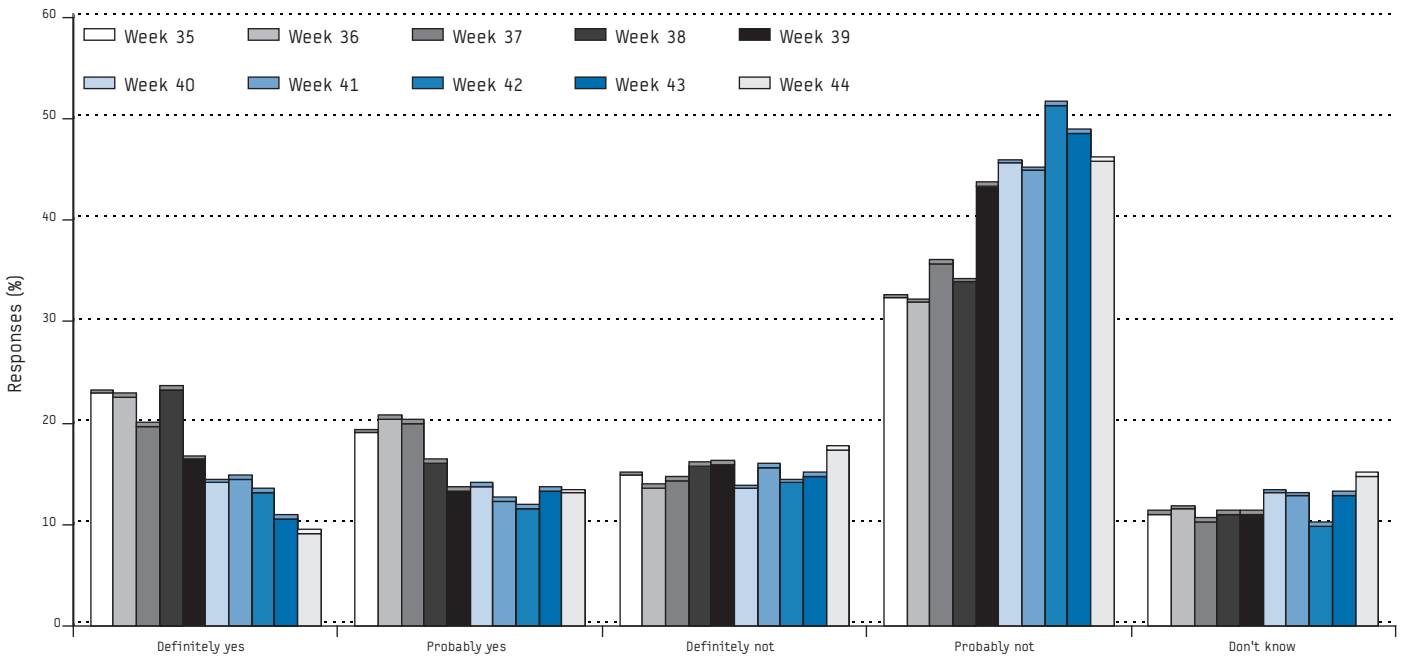



**Table 1**

**Univariate association of variables potentially affecting respondents' intentions concerning vaccination, Greece, 2009**

| | Intention to accept vaccination | | | | | |
|---|---|---|---|---|---|---|
| | Definitely yes | Probably yes | Probably not | Definitely not | Don't know | P value |
| Age, mean | 56.7 (17.0) | 49.3 (19.3) | 52.4 (16.9) | 49.8 (15.8) | 56.8 (17.3) | <0.001 |
| Sex | | | | | | |
| Male | 50 (14.6) | 62 (18.1) | 60 (20.2) | 117 (34.2) | 44 (12.9) | <0.001 |
| Female | 41 (6.2) | 69 (10.5) | 104 (15.8) | 341 (51.8) | 103 (15.7) | |
| Urban/rural location | | | | | | |
| Athens/Thessaloniki | 41 (8.2) | 61 (12.2) | 84 (16.8) | 250 (50.0) | 64 (12.8) | 0.196 |
| Other urban | 21 (8.4) | 36 (14.4) | 49 (19.6) | 106 (42.4) | 38 (15.2) | |
| Semi-rural/rural | 29 (11.6) | 34 (13.6) | 40 (16.0) | 102 (40.8) | 45 (18.0) | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Educational attainment | | | | | | |
| Primary school | 20 (7.4) | 34 (12.6) | 53 (19.6) | 101 (37.4) | 62 (23.0) | |
| High school (3 years) | 11 (10.8) | 16 (15.7) | 16 (15.7) | 36 (35.3) | 23 (22.6) | <0.001 |
| High school (6 years) | 32 (10.2) | 39 (12.4) | 47 (14.9) | 162 (51.4) | 35 (11.1) | |
| University/postgraduate studies | 28 (9.0) | 42 (13.4) | 57 (18.2) | 159 (50.8) | 27 (8.6) | |
| Presence of chronic illness (respondent) | | | | | | |
| No | 65 (8.3) | 97 (12.3) | 138 (17.6) | 379 (48.2) | 107 (13.6) | 0.016 |
| Yes | 26 (12.2) | 34 (15.9) | 35 (16.4) | 79 (36.9) | 40 (18.7) | |
| Presence of chronic illness (household) | | | | | | |
| No | 54 (8.2) | 75 (11.4) | 116 (17.7) | 325 (49.5) | 87 (13.2) | 0.005 |
| Yes | 37 (10.4) | 56 (16.4) | 56 (16.4) | 132 (38.7) | 60 (17.6) | |
| Children aged 0-12 years in the household | | | | | | |
| No | 77 (9.9) | 110 (14.1) | 139 (17.9) | 332 (42.7) | 120 (15.4) | 0.005 |
| Yes | 14 (6.3) | 21 (9.5) | 34 (15.3) | 126 (56.8) | 27 (12.2) | |
| Persons ≥65 years in the household | | | | | | 0.003 |
| No | 45 (7.2) | 81 (13.0) | 113 (18.1) | 306 (49.1) | 78 (12.5) | |
| Yes | 46 (12.2) | 50 (13.3) | 60 (15.9) | 152 (40.3) | 69 (18.3) | |
| Pregnant women (respondent) | | | | | | |
| No | 90 (9.1) | 130 (13.1) | 173 (17.5) | 451 (45.6) | 146 (14.8) | 0.513 |
| Yes | 1 (10.0) | 1 (10.0) | 0 (0.0) | 7 (70.0) | 1 (10.0) | |
| Pregnant women (household) | | | | | | |
| No | 90 (9.2) | 129 (13.2) | 171 (17.4) | 445 (45.4) | 146 (14.9) | 0.372 |
| Yes | 1 (5.3) | 2 (10.5) | 2 (10.5) | 13 (68.4) | 10 (5.3) | |
| Seasonal vaccination (respondent) | | | | | | |
| No | 61 (7.5) | 102 (12.6) | 146 (18.0) | 399 (49.2) | 103 (12.7) | <0.001 |
| Yes | 30 (15.9) | 29 (15.3) | 27 (14.3) | 59 (31.2) | 44 (23.3) | |
| Seasonal vaccination (household) | | | | | | |
| No | 56 (7.6) | 97 (13.1) | 138 (18.6) | 359 (48.5) | 91 (12.3) | <0.001 |
| Yes | 35 (13.5) | 34 (13.1) | 35 (13.5) | 99 (38.2) | 56 (21.6) | |
| Self-reported level of knowledge about pandemic influenza | | | | | | |
| Very much | 16 (10.1) | 18 (11.3) | 26 (16.4) | 78 (49.1) | 21 (13.2) | |
| Quite enough | 46 (7.7) | 76 (12.8) | 103 (17.3) | 284 (47.8) | 85 (14.3) | 0.705 |
| Little | 24 (12.2) | 29 (14.7) | 35 (17.8) | 76 (38.9) | 33 (16.8) | |
| Not at all | 5 (12.2) | 6 (14.6) | 7 (17.1) | 17 (41.5) | 6 (14.6) | |
| Likelihood of getting infected | | | | | | |
| Very likely | 9 (10.0) | 10 (11.1) | 16 (17.8) | 44 (48.9) | 11 (12.2) | |
| Quite likely | 16 (9.1) | 26 (14.8) | 32 (18.2) | 83 (47.2) | 19 (10.8) | 0.472 |
| Not very likely | 26 (8.3) | 47 (14.9) | 59 (18.7) | 145 (46.0) | 38 (12.1) | |
| Not likely at all | 13 (7.0) | 17 (9.1) | 24 (12.8) | 110 (58.8) | 23 (12.3) | |
| If likely to become infected, perceptions related to severity | | | | | | |
| High risk | 15 (19.2) | 9 (11.5) | 9 (11.5) | 30 (38.5) | 15 (19.2) | |
| Moderate risk | 19 (11.2) | 31 (18.3) | 35 (20.7) | 58 (34.3) | 25 (15.4) | <0.001 |
| Little risk | 10 (4.3) | 30 (12.8) | 43 (18.4) | 131 (56.0) | 20 (8.6) | |
| No risk | 2 (3.6) | 3 (5.4) | 12 (21.4) | 37 (66.1) | 2 (3.6) | |

Values express number of respondents and brackets indicate the corresponding percentage with the exception of age where mean (standard deviation) are provided.

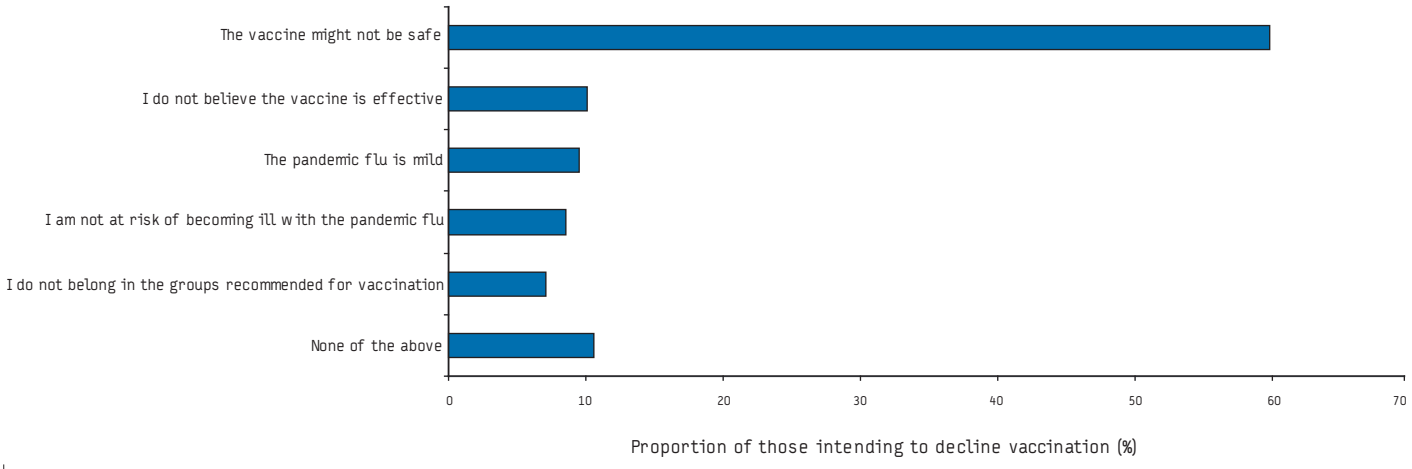
## Table 2

**Multiple logistic regression models for intention to decline pandemic vaccination (versus those intending to accept), Greece, 2009**

| Variable | odds ratio | 95% confidence interval | P value |
|---|---|---|---|
| Sex | | | |
| Male | 1 | | |
| Female | 2.75 | (1.94 to 3.90) | <0.001 |
| Age (years) | | | |
| 15-29 | 1 | | |
| 30-64 | 1.85 | (1.07 to 3.22) | 0.029 |
| 65+ | 1.24 | (0.54 to 2.81) | 0.612 |
| Educational attainment | | | |
| Nine-year high school/university | 1 | | |
| Primary/three-year high school | 1.07 | (0.71 to 1.60) | 0.751 |
| Urban/rural location | | | |
| Semi-rural/rural | 1 | | |
| Athens/Thessaloniki | 1.56 | (1.02 to 2.38) | 0.040 |
| Other urban | 1.22 | (0.75 to 1.96) | 0.424 |
| Presence of chronic illness (household) | | | |
| Yes | 1 | | |
| No | 1.60 | (1.10 to 2.33) | 0.013 |
| Presence of child aged 0-12 years in household | | | |
| Yes | 1 | | |
| No | 1.47 | (0.91 to 2.38) | 0.118 |
| Vaccination for seasonal influenza in the household | | | |
| Yes | 1 | | |
| No | 1.46 | (0.96 to 2.23) | 0.077 |
| Person ≥65 years in the household | | | |
| Yes | 1 | | |
| No | 1.17 | (0.65 to 2.09) | 0.600 |
| Self-reported level of knowledge about pandemic influenza | | | |
| Not at all/little | 1 | | |
| Quite enough/very much | 1.30 | (0.87 to 1.93) | 0.196 |
| Likely to get infected and perceived severity | | | |
| Likely and dangerous | 1 | | |
| Likely but not dangerous | 2.72 | (1.73 to 4.27) | <0.001 |
| Not likely at all | 3.26 | (1.92 to 5.53) | <0.001 |
| Don't know if likely | 1.36 | (0.84 to 2.18) | 0.210 |

## Figure 2

**Reasons for intention to decline pandemic vaccination as reported by 631 participants in week 44/2009 (multiple answers were allowed), Greece**



received that vaccine before (45.5% versus 67.2%). It is of note that more than half of the respondents with chronic conditions (53.3%) did not intend to accept pandemic vaccination ("probably not/definitely not") and seven of the 10 pregnant women in the sample provided "definitely not" as an answer.

According to multiple logistic regression analysis, respondents who did not intend to get vaccinated were more often found among females (odds ratio (OR) versus males: 2.75, 95% confidence interval (CI): 1.94 to 3.90, p<0.001), among individuals aged 30-64 years (OR versus 15-29 year-olds: 1.85, 95% CI: 1.07 to 3.22, p=0.029), among those with a perception of low likelihood of getting infected or low risk associated with it (OR (95% CI) compared to those reporting "likely of getting infected and dangerous": 2.72 (1.73 to 4.27) for those answering "likely but not dangerous" and 3.26 (1.92 to 5.53) for those reporting "not likely at all", p<0.001) (Table 2). Additionally, participants from households where no member suffered from chronic illnesses were more likely to provide negative answers concerning vaccination (OR 1.60 versus households with members suffering from chronic illness, 95% CI: 1.10 to 2.13, p=0.013). A multiple logistic regression model was used to identify factors associated with higher probability of answering "I don't know" compared to "probably yes/definitely yes". Females and individuals reporting a low educational status of the head of their household were more likely to be undecided whether to get vaccinated or not (females versus males: OR=2.42, 95% CI: 1.50 to 3.93, p<0.001 and primary/three-year high school versus nine-year high school/university: OR=2.24, 95% CI: 1.35 to 3.73, p=0.002).

In week 44, 631 participants who indicated "probably not" or "definitely not" as their intention to get vaccinated were further asked to indicate their reasons among a pre-defined set of possible answers (multiple answers were allowed) (Figure 2). For the vast majority of the respondents (59.8%), the main reason was their belief that the vaccine might not be safe.

## Discussion

According to our findings, the intention to decline vaccination against pandemic influenza A(H1N1) showed increasing trends since the end of August 2009 and reached 63% in week 44 (26 October-1 November 2009). The corresponding rate of likely acceptance in week 44 was 22.2%, whereas a considerable proportion of the population (15%) had not decided yet. Vaccination had not started in Greece at that time. The most frequently reported barrier against the uptake of vaccination was the fear that the vaccine might not be safe. It is noteworthy that the rates of intention to decline among individuals belonging to vaccination target groups were high: 53.3% among people with chronic conditions and 70.0% in a small sample of pregnant women. Factors independently associated with intention to decline vaccination were female sex, age between 30 and 64 years, perception of low likelihood of getting infected or of low risk associated with it, and absence of household members suffering from chronic illnesses.

To our knowledge, this is the only study conducted so far in a European population during the ongoing influenza A(H1N1) pandemic that assesses perceptions towards influenza, willingness to accept vaccination and related barriers in vaccine uptake. The sample was large (1,000 households per week) and representative of Greek households with quotas based on household size and urban/rural location. Data was collected on numerous items that allowed identifying the profile of the population that will be less likely to accept vaccination. It should be taken into account that as an epidemic unfolds in a population, intentions may change. Other factors, such as media attention or vaccine promotion programmes, may also play a role in shaping perceptions and attitudes. These may differ from country to country and as a result, our estimates concerning willingness to accept vaccination might not strictly apply in the case of other populations. However, as those who do not wish to get vaccinated may have similar characteristics in all countries, qualitative results concerning attitudinal barriers could be used to explain negative intentions towards vaccine uptake in other countries too.

Low rates of intention to accept vaccination have also been reported by other studies on the current pandemic or pre-pandemic vaccines [4-7]. As in our study, perceptions concerning the risk associated with infection were consistently found to affect the intention to accept or decline vaccination and the fear of side-effects was the most frequently reported barrier [6,7]. Even in the case of seasonal influenza, concerns about side effects were reported at high rates (43%) as a reason for avoiding immunization [8].

Overall, this study has identified high rates of intention to decline pandemic vaccination in the Greek population, even among vaccination target groups, mainly due to the perception that the vaccine might not be safe. Vaccination promotion programmes should be carefully designed in order to achieve timely vaccination of the target populations at satisfactory levels of coverage.

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# Behaviours regarding preventive measures against pandemic H1N1 influenza among Italian healthcare workers, October 2009

**G La Torre (giuseppe.latorre@uniroma.it)[1], D Di Thiene[1], C Cadeddu[2], W Ricciardi[2], A Boccia[1]**
1. Clinical Medicine and Public Health Unit, Sapienza University of Rome, Italy
2. Institute of Hygiene, Catholic University of the Sacred Heart Rome, Italy

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A survey on attitudes and behaviours towards preventive measures against pandemic H1N1 influenza 2009 was carried out during the month of October 2009 in Italy through an online questionnaire adapted to the Italian situation from a similar survey of the Harvard School of Public Health in the United States (US). Results show that the intention to get vaccinated against pandemic H1N1 influenza 2009 is generally low and that there are differences in attitudes and behaviours towards preventive measures against pandemic H1N1 influenza 2009 between physicians and nurses, especially concerning vaccination. Differences relate also to sex, region of residence and marital status.

## Introduction

One of the main concerns related to the present pandemic H1N1 influenza 2009 is the overwhelming burden on medical structures and resources that it poses and the consequent negative impact on mortality and morbidity. This situation puts healthcare workers (HCW) in the unusual position of being both the main actors and one of the main targets of the prevention strategies against the pandemic H1N1 influenza 2009, and considering also their usual unavoidable risk of being an important vector for transmission [1,2]. That is why it is so important to understand the behaviour and attitudes of HCW in relation to the spreading pandemic [3].The importance of this understanding is also demonstrated by studies carried out worldwide [4,5].

The aim of our survey was to gather information about attitudes and behaviours towards preventive measures against pandemic influenza among Italian HCW, taking into account the characteristics of the Italian health care setting. The survey was carried out by means of a questionnaire distributed to and collected from physicians and nurses.

## Materials and methods

The questionnaire was designed by the Clinical Medicine and Public Health section of the Sapienza University of Rome, adapted to the Italian situation on the basis of a similar one used in a telephone survey in the US by the Harvard School of Public Health [6]. The adaptation consisted in changing some questions, i.e. concerning health insurance (Italy has a National Health System) or referring to pandemic H1N1 influenza 2009 instead of swine influenza as in the original version of the questionnaire.

The questionnaire was made available through the Italian Journal of Public Health website (www.ijph.it) and a remote recording

**Table 1**

**Socio-demographical characteristics of the survey participants, Italy, October 2009 (n=1,960)**

| Socio-demographical characteristics (number of responders) | Total |
|---|---|
| | |
| **Age group (n=1,960)** | |
| 18-29 years | 82 (4.2%) |
| 30-49 years | 1,444 (73.7%) |
| 50-64 years | 422 (21.5%) |
| ≥ 65 years | 12 (0.6%) |
| | |
| **Sex (n=1,960)** | |
| Female | 1,360 (69.4%) |
| Male | 600 (30.6%) |
| | |
| **Civil status (n=1,908)** | |
| Married/cohabitant | 1,480 (78%) |
| Single | 264 (13.7%) |
| Separated/divorced | 144 (7.3%) |
| Widow | 20 (1%) |
| | |
| **Children < 18 years in your home (n=1,955)** | |
| Yes | 1,007 (51.5%) |
| No | 948 (48.5%) |
| | |
| **Job (n=1,960)** | |
| Physicians | 249 (12.7%) |
| Nurses | 1,711 (87.3%) |
| | |
| **Regions of residence (n=1,955)** | |
| Northern Italy | 1,101 (56.2%) |
| Central Italy | 598 (30.5%) |
| Southern Italy and islands | 256 (13.1%) |
| | |
| **Health status (n=1,960)** | |
| Excellent, very good, good | 1,874 (95.6%) |
| Poor | 86 (4.4%) |

system collected the anonymous answers given by physicians and nurses [8]. The survey was advertised through an email sent to addresses in databases of Public Health professionals and nurses, owned by the Italian National Society of Public Health. Access to the online questionnaire was permitted from 1 to 31 October 2009, including week-ends when the website was accessed more often.

In order to perform an inferential analysis, we considered the following dependent variables:

a) willingness to get vaccinated against pandemic H1N1 influenza 2009;

b) washing hands and using hand sanitisers more frequently than before the beginning of the pandemic.

A univariate analysis was then carried out using a chi-squared test in order to investigate the association between the dependent variables and socio-demographic characteristics, as well as occupation. Moreover, two multiple logistic regression analyses were performed, using the backward elimination procedure as described by Hosmer and Lemeshow [7]. The goodness of fit of the regression model was tested using the Hosmer-Lemeshow test. The following were considered as potential explanatory variables: age group (18-29 years as the reference group), sex (reference modality male),

**Table 2**

**Univariate analysis to investigate the association between the dependent variables and socio-demographic characteristics, as well as occupation, Italy, October 2009 (n=1,960)**

| | Would you get vaccinated against pandemic influenza ? | | | Did you wash your hand or use hand sanitiser more frequently ? | | |
|---|---|---|---|---|---|---|
| | Yes | No | p | Yes | No | p |
| **Age group** | | | | | | |
| 18-29 | 26 (41.3%) | 37 (58.7%) | <0.001 | 56 (68.3%) | 26 (31.7%) | 0.068 |
| 30-49 | 359 (31.1%) | 797 (68.9%) | | 1,112 (77.5%) | 323 (22.5%) | |
| ≥ 50 | 179 (50.1%) | 178 (49.9%) | | 345 (79.9%) | 87 (20.1%) | |
| **Sex** | | | | | | |
| Male | 244 (49.2%) | 252 (50.8%) | <0.001 | 429 (72.2%) | 165 (27.8%) | <0.001 |
| Female | 320 (29.6%) | 760 (70.4%) | | 1084 (80%) | 271 (20%) | |
| **Residence** | | | | | | |
| Northern Italy | 261 (29.1%) | 637 (70.9%) | <0.001 | 837 (76.5%) | 257 (23.5%) | 0.267 |
| Central Italy | 187 (39.1%) | 291 (60.9%) | | 471 (78.9%) | 126 (21.1%) | |
| Southern Italy and islands | 115 (58.4%) | 82 (41.6%) | | 204 (80.6%) | 49 (19.4%) | |
| **Marital status** | | | | | | |
| Married/cohabitant | 438 (36.4%) | 765 (63.6%) | 0.355 | 1,169 (79.4%) | 303 (20.6%) | 0.001 |
| Single/divorced/ separated/ widow | 126 (33.8%) | 247 (66.2%) | | 344 (72.1%) | 133 (27.9%) | |
| **Occupation** | | | | | | |
| Physicians | 141 (67.1%) | 69 (32.9%) | <0.001 | 161 (64.7%) | 88 (35.3%) | <0.001 |
| Nurses | 423 (31%) | 943 (69%) | | 1,352 (79.5%) | 348 (20.5%) | |

**Table 3**

**Multivariate analysis, Italy, October 2009 (n=1,908)**

| | Yes, I would get vaccinated | | Yes, I washed my hands or used hand sanitisers more frequently | |
|---|---|---|---|---|
| | Crude OR (IC95%) | Adjusted OR (IC95%) | Crude OR (IC95%) | Adjusted OR (IC95%) |
| **Age group** | | | | |
| 18-29 (reference) | 1 | 1 | 1 | 1 |
| 30-49 | 0.71 (0.44-1.15) | 0.66 (0.52-0.83) | 1.6 (0.99-2.59) | - |
| ≥ 50 | 1.51 (0.91-2.5) | | 1.84 (1.09-3.1) | 1.56 (1.17-2.08) |
| **Sex** | | | | |
| Male (reference) | 1 | 1 | 1 | 1 |
| Female | 0.45 (0.37-0.55) | 0.64 (0.51-0.8) | 1.54 (1.23-1.92) | 1.59 (1.24-2.03) |
| **Region of residence** | | | | |
| Northern Italy (reference) | 1 | 1 | 1 | 1 |
| Central Italy | 1.47 (1.17-1.83) | - | 1.16(0.92-1.48) | 1.36 (1.06-1.76) |
| Southern Italy and islands | 2.63 (1.98-3.49) | 1.81 (1.36-2.41) | 1.3 (0.92-1.82) | 1.76 (1.23-2.53) |
| **Marital status** | | | | |
| Single/divorced/separated/widow (reference) | 1 | | 1 | 1 |
| Married/cohabitant | 1.18 (0.94-1.49) | | 1.49 (1.18-1.89) | 1.54 (1.21-1.96) |
| **Occupation** | | | | |
| Nurses (reference) | 1 | 1 | 1 | 1 |
| Physicians | 3.98 (3.02-5.23) | 2.87 (2.14-3.85) | 0.47 (0.35-0.63) | 0.42 (0.3-0.57) |
| **p-value from Hosmer-Lemeshow test** | | 0.52 | | 0.58 |

OR: odds ratio; CI: confidence interval

region of residence (reference modality Northern Italy), marital status (single/divorced/separated/widow as the reference group), occupation (physicians vs. nurses, with the latter as the reference group). The level of statistical significance was set at a p-value of $\leq 0.05$.

The statistical analysis was performed using the statistical software SPSS 13.0 for Windows.

## Results

One thousand nine hundred and sixty individuals participated in the survey (249 physicians, 12.7%, and 1,711 nurses, 87.3%). The socio-demographical characteristics of the sample are shown in Table 1.

We found that 70.4% of the 1,360 females of our sample would not get vaccinated against pandemic H1N1 influenza 2009, while 49.2% of the 600 males would get vaccinated ($p<0.001$) (Table 2). The main difference for the same question was related to occupation: 67% of physicians and 31% of nurses would get vaccinated against pandemic H1N1 influenza 2009 ($p<0.001$). In contrast, nurses were more prone (79.5%) than physicians (64.7%) to wash their hands or use hand sanitisers more frequently in response to reports of pandemic influenza ($p<0.001$).

Results from the multivariate analysis (Table 3) show that respondents aged 30-49 years are less likely to get vaccinated in comparison to young adults (18-29 years old) (adjusted odds ratio (AOR)=0.66; 95% confidence interval (CI): 0.52-0.83). Females also are less likely to get vaccinated (AOR=0.64; 95%CI: 0.51-0.8), confirming the results from the univariate analysis. Health professionals who are more likely to get vaccinated live in Southern Italy or on the islands (AOR=1.81; 95%CI: 1.36-2.41) and are physicians (AOR=2.87; 95%CI: 2.14-3.85).

As far as concerns the variable "Yes, I washed my hands or used hand sanitisers more frequently", there is a statistically significant association with: age (≥50 years: AOR=1.56; 95%CI: 1.17-2.08), sex (female: AOR=1.59; 95% CI: 1.24-2.03), region of residence (Central Italy: AOR=1.36; 95%CI: 1.06-1.76; Southern Italy and islands: AOR=1.76; 95%CI: 1.23-2.53), marital status (married/cohabitant: AOR=1.54; 95%CI: 1.21-1.96) and occupation (physicians: AOR=0.42; 95%CI: 0.3-0.57).

## Conclusions

HCW are a strategic target for pandemic H1N1 influenza 2009 prevention such as vaccination and frequent hand-washing, since they are at higher risk themselves of contracting influenza, can place their patients at risk and are critical for a functioning health care system. Our online survey demonstrated that pandemic H1N1 influenza 2009 modified the behaviour of HCW, but a high percentage may still not realise that vaccination is a fundamental means of prevention and how important it is that they get vaccinated. This finding is surprising, as many studies worldwide present different attitudes among HCW [1,2].

The present study has some limitations, and the results must be interpreted with caution. First of all, a possible selection bias could have occurred, since healthcare professionals with internet skills would have been more likely to participate in the online survey. Moreover, it is likely that participants are mainly representative of younger HCW and this is supported by the age of responders (almost half of the participants should have been over 50 years old, according to the information included in the databases). Concerning possible information bias, we are convinced of the validity of the self-report answers, since it is unlikely that participants spent time giving unreliable and biased views of their attitudes and behaviours.

Despite some limitations, our survey could be a useful tool for Italian decision makers to promote and launch programmes and campaigns aimed at informing and educating HCW. The results could also be used to motivate HCW to adopt attitudes and decisions which correspond to public health policies, since at the end of November 2009, only 14% of healthcare professionals had been vaccinated against pandemic H1N1 influenza 2009 at the national level [8]. Finally, this study could also help tailor vaccination campaigns by concentrating on groups (nurses, females, adults ≥30 years) or regions (Northern Italy) where the intended vaccine uptake is lower.

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# Behaviour of the pandemic H1N1 influenza virus in Andalusia, Spain, at the onset of the 2009-10 season

**J M Mayoral Cortés1, L Puell Gómez (luz.puell.ext@juntadeandalucia.es)[1], E Pérez Morilla[1], V Gallardo García[1], E Duran Pla[1], J C Fernandez Merino[1], J Guillén Enriquez[1], J C Carmona[1], G Andérica[1], I Mateos[1], J M Navarro Marí[2], M Pérez Ruiz[2], A Daponte[3]**

1. Epidemiology Department, Regional Ministry of Health, Andalusian Regional Government, Spain
2. Influenza Reference Laboratory, Virgen de las Nieves Hospital, Granada, Spain
3. Public Health and Health Protection Area, Andalusian Public Health School, Granada, Spain

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In Andalusia, Spain, the pandemic influenza A(H1N1)v virus has spread throughout the community, being the dominant influenza strain in the season so far. The current objective of the Andalusia Health Service is focussed on the mitigation of the health and social impact by appropriate care of the patients at home or in health centres. The 2009-10 seasonal influenza epidemic started early compared with to previous seasons. This article analyses the influenza A(H1N1)v situation in Andalusia until the week 39/2009.

## Introduction

In Spain, first suspected cases of pandemic H1N1 influenza were notified on 26 April 2009. Starting with these first cases, an epidemic outbreak of a holomiantic nature was seen in Andalusia, with the primary cases in students who had travelled to Mexico and secondary cases in their families. There were 44 confirmed cases in this first epidemic wave until 5 May 2009. The average age of the cases was 24 years (range: 14-55 years). In 42 of them, the main symptoms were fever and cough, and 18 also had diarrhoea. All of them had mild clinical signs without complications [1].

During the first days of the outbreak, contingency plans were set up based on epidemiological surveillance, and outbreak control measures were adopted through early alert and rapid response systems. Protocols integrated the activities of the public health services, healthcare services, and the influenza reference laboratories [2]. The objective was initially to slow down the propagation of infection by identifying cases according to clinical and epidemiological criteria, reporting the first generation imported cases, their treatment, the measures adopted to prevent secondary cases and outbreaks, with an active search for any contacts. As a preventive measure, cases and contacts received treatment with oseltamivir with the recommendation to remain at home.

The declaration of pandemic phase 6 by the World Health Organization (WHO) on 11 June 2009 [3] indicated that it was no longer feasible to stop the spread of the new influenza virus. Since then, the epidemiological surveillance strategies have been aimed at defining scenarios that could aid healthcare services to respond to this emergency in order to reduce transmission and the number of affected people, and to identify and protect the most vulnerable population groups.

## Surveillance of influenza in Andalusia

The epidemiological and virological surveillance of influenza in Andalusia is carried out through the Medical Sentinel Network of the Andalusian Epidemiological Surveillance System (SVEA), which consists of 128 sentinel physicians chosen according to population distribution, who are based in primary healthcare centres and cover 170,668 inhabitants (2.08% of the Andalusian population). The influenza reference laboratory, located at the 'Virgen de las Nieves' hospital in Granada, is part of this network.

The surveillance of severe cases is undertaken through the SVEA, by means of individualised notification of the cases admitted to the public hospitals of Andalusia. Information about the use of emergency services was also collected from the computerised emergency records of public hospitals.

A case of influenza was defined as established by the European Centre for Disease Prevention and Control (ECDC) [4]. The presence of influenza A(H1N1)v was confirmed by realtime PCR carried out with SW H1 forward and SW H1 reverse primers and Taqman SW H1 probe targeted at the H1 gene of this virus, as recommended [5].

## Characteristics of pandemic H1N1 influenza cases in Andalusia

After the first pandemic wave in April and May 2009, the influenza activity in Andalusia decreased before the summer. New cases were seen in week 28 (beginning 6 July) and increased until week 39 (beginning 21 September), when the registered influenza incidence reached 147 cases per 100,000 inhabitants (Figure 1).

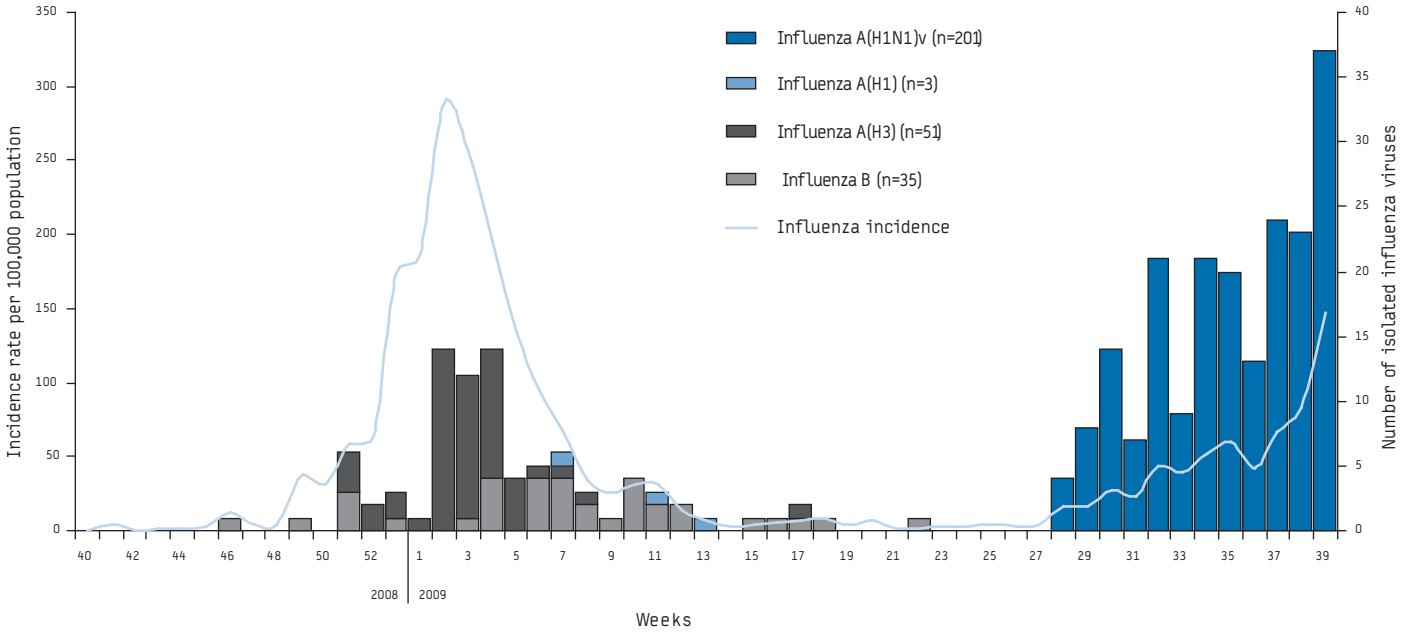
In weeks 38 and 39/2009, the incidence was higher than the epidemic threshold, established as 64.1 cases per 100,000. That implies a widespread dissemination of influenza within the population, two months ahead of the usual period for seasonal influenza. The increased influenza activity in this period is associated with a widespread escalation of the circulation of the

influenza A(H1N1)v virus. Since week 28, all circulating influenza viruses have been sub-typed and identified as influenza A(H1N1) v (see Figure 1).

The cumulative incidence rate for influenza until week 39 week was 643 cases per 100,000 inhabitants. In that week the highest rates were registered in the age group of 5-14 year-olds with 132 cases per 100,000, followed by the under five year-olds with 28 cases per 100,000. The incidence rate for those over 64 years of age was six cases per 100,000. Almost all cases showed mild symptoms lasting for a few days and responded to antipyretic

**Figure 1**

**Weekly influenza incidence rate and isolated influenza viruses, Andalusia, 2008-9 and 2009-10 seasons**




**Table**

**Characteristics of severe cases of influenza A(H1N1)v, Andalusia, weeks 17-39/2009**

| | All hospitalised cases | Hospitalised cases <15 years old | Cases admitted to intensive care unit |
|---|---|---|---|
| **Number of cases** | **311** | **41** | **28** |
| Age (years) | Mean: 35.05<br>Median: 32<br>Range: 2-90 | Mean: 8.56<br>Median: 9<br>Range: 2-15 | Mean: 35.05<br>Median: 33.5<br>Range: 2-77 |
| Sex | Male: 129 (41.5%)<br>Female: 182 (58.5%) | Male: 26 (63.4%) Female: 15 (36.6%) | Male: 8 (28.6%)<br>Female: 20 (71.4%) |
| **Risk factors** | **N** | **N** | **N** |
| Asthma | 21 | 4 | 2 |
| Cancer | 10 | 1 | 1 |
| Cardiopathy | 24 | 2 | 0 |
| Diabetes | 18 | 0 | 4 |
| Chronic hepatic disease | 1 | 1 | 0 |
| Active immunodeficiency | 17 | 2 | 2 |
| Obesity (body mass index ≥40) | 8 | 1 | 1 |
| Chronic respiratory disease | 31 | 5 | 7 |
| Convulsive disorders | 4 | 1 | 0 |
| Renal failure | 2 | 1 | 2 |
| Other metabolic diseases | 2 | 2 | 1 |
| Other risk factors | 26 | 4 | 2 |
| No risk factors | 3 | 0 | 1 |
| **No information** | **171 (55%)** | **22 (53.7%)** | **12 (43%)** |

treatment. The most frequent symptoms were fever and cough in 94% and 88% of the cases, respectively.

In the study period, 311 of the confirmed cases notified in Andalusia were severe and required hospitalisation. Of those, 28 (9%) were admitted to intensive care units (ICUs). The hospitalisation rate for influenza was 3.7 per 100,000 inhabitants. Males represented 41% of the hospitalised cases, and 59% were female, a male/female ratio of 0.69.The age of the hospitalised cases ranged between 2 and 86 years, and 92% were under 65 years old. The average age of the cases admitted to ICUs was 38 years, with a median age of 35 years.

The most frequently registered complication during the course of the disease in severely ill patients was primary viral pneumonia, in 120 cases (39%). About 75% of them were 15 to 59 years of age.

For 137 of the 311 hospitalised cases (44%), information on risk factors was recorded (see Table). Main risk factors were: prior pulmonary pathology (especially asthma or chronic obstructive pulmonary disease) in 38% of them, a history of cardiovascular disease (18%), immunodeficiency (12%), diabetes (13%), cancer (7%), morbid obesity (6%), and convulsive disorders (3%).

Forty-one of the 311 hospitalised cases were under 15 years of age. Information on risk factors was recorded for 19 of them. Fifteen (80%) presented at least one risk factor (mainly asthma and other chronic pulmonary diseases).

Of the 28 cases admitted to ICUs (including adults and children), information on risk factors was obtained for 20 cases (Table 1). The most common factors were prior pulmonary pathology (chronic respiratory disease or asthma) in eight cases and diabetes in four cases. One case did not present any risk factor.

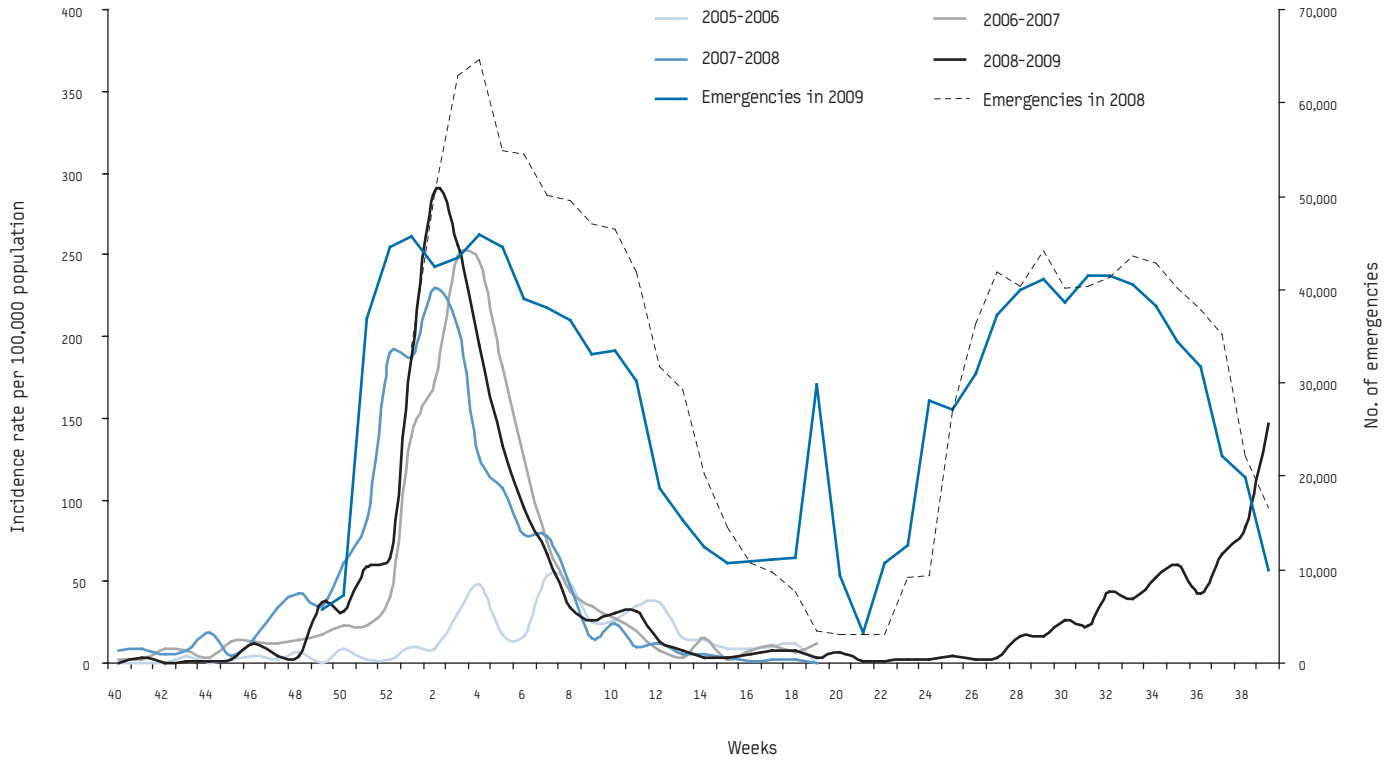
In the same period, 13 deaths due to influenza A(H1N1)v were registered in Andalusia. The estimated death rate was 0.02%. The average age of death was 44.3 years (range: 9 85 years). Information about risk factors was recorded in 10 of them. They were prior pulmonary pathology (especially chronic obstructive pulmonary disease), diabetes, morbid obesity (body mass index ≥40), renal failure, convulsive disorders and cardiopathy.

For 75 of all hospitalised cases, we had information on the time from beginning of symptoms to start of treatment. The median time was three days (range 0-24 days). This delay increased with the severity of cases: a median of three days for the 66 hospitalised, and of four days for the nine cases admitted to ICUs.

The impact of the H1N1 influenza pandemic on the health services in Andalusia was most obvious at the beginning, between weeks 17 (beginning 20 April) and 21/2009. Attendance of hospital emergency departments peaked during this period (Figure 2). This peak in emergencies represented the alarm the first cases of pandemic H1N1 influenza caused in the population and did not reflect the number of notified cases during this outbreak. The containment measures undertaken, together with environmental factors (increased temperatures), and a reduction in the flow of travellers returning from Mexico, contributed to the control of the first phase of the outbreak. From week 22 to week 39/2009, the frequency of emergencies was similar to that observed in the

**Figure 2**

**Influenza incidence rate and hospital emergencies in Andalusia, 2005-2009**



previous year, despite the increase in the incidence of influenza that took place after week 28.

## Conclusions

Most cases of influenza caused by the pandemic influenza A(H1N1)v virus presented with a mild clinical picture similar to seasonal influenza. The majority of cases occurred in children of school age and in adults under 65 years of age, with the highest frequency of severe and fatal cases found in young adults. A significant proportion of those presented risk factors such as chronic pulmonary pathologies, cardiopathy, diabetes and morbid obesity. Similar results were observed in rest of Spain in the same period [1,2]. It was observed that a delay in the start of treatment increased the severity of the cases.

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# Detection of human norovirus from frozen raspberries in a cluster of gastroenteritis outbreaks

**L Maunula (leena.maunula@helsinki.fi)[1], M Roivainen[2], M Keränen[3], S Mäkelä[4], K Söderberg[1], M Summa[1], C H von Bonsdorff[1], M Lappalainen[5], T Korhonen[2], M Kuusi[2], T Niskanen[6]**

1. Department of Food and Environmental Hygiene, University of Helsinki, Finland
2. Institute of Health and Welfare, Helsinki. Finland
3. Regional Food Inspection Authority, Lahti, Finland
4. Regional Food Inspection Authority, Social and Health Care Group, Päijät-Häme, Finland
5. Laboratory Division (HUSLAB), Department of Virology, Helsinki University Central Hospital, Helsinki, Finland
6. Finnish Food Safety Authority Evira, Helsinki, Finland

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We describe a cluster of norovirus outbreaks affecting about 200 people in Southern Finland in September and October 2009. All outbreaks occurred after consumption of imported raspberries from the same batch intended for the catering sector. Human norovirus genotype GI.4 was found in frozen raspberries. The berries were served in toppings of cakes in separate catering settings or mixed in curd cheese as a snack for children in a daycare center. The relative risk for consumption of the berry dish was 3.0 ($p \leq 0.05$) at the daycare centre. Human norovirus GI.4 was also detected in samples from two patients, and in berries. Both shared identical partial capsid sequences. Based on the results of epidemiological, trace-back and laboratory investigations it was concluded that one particular batch of frozen raspberries was the source of all outbreaks.

## Introduction

Human norovirus is a common cause of outbreaks of acute gastroenteritis worldwide [1]. Food-borne outbreaks caused by contaminated shellfish occur commonly, but fresh products, especially raspberries have also been found to be the vehicle [2, 3, 4]. In Finland, from 1997 to 1999, several norovirus outbreaks were linked to imported frozen raspberries [2,5]. It still remains unclear how the berries were contaminated, but it seemed to have occurred already in the countries of origin. Pre-harvest irrigation or hygiene failures during harvest/freezing have been suggested as possible sources of contamination [6]. A proper heating of frozen raspberries prior to consumption has been recommended by the Finnish Food Safety Authority since 2000 but it is occasionally neglected.

Here we describe a trace-back investigation in a cluster of three food-borne norovirus outbreaks linked to consumption of imported raspberries affecting about 200 people in Southern Finland in September and October 2009. The epidemiological investigation was performed of one of the outbreaks, at a daycare centre.

## Outbreak in the daycare centre

A curd cheese dish mixed with raspberries (originally frozen) was served without heating the berries and eaten by about 90 persons (majority children, less than 7 years old) at a daycare centre on 2 October 2009 at 2-2.30 pm. On Saturday evening, 3 October, more than 20 of the 90 persons started symptoms of vomiting and diarrhoea (Table 1). The food inspection authorities were informed about the outbreak on 6 October and started an epidemiological investigation. No samples of the dish were available for investigation but the remaining frozen raspberries were sent for bacteriological and virological examination on 7 October. Also samples from patients were collected, and questionnaires were distributed to the children's parents and the personnel on 7 October to investigate the possible source of the outbreak. The outbreak occurred at a daycare centre in a city of 100,700 inhabitants in Southern Finland.

## Epidemiological analysis

Questionnaires were obtained from 69 people at the daycare centre. A case was defined as a person who was working, or at daycare at the daycare centre, and fell ill with vomiting and diarrhoea between 2 and 5 October 2009. A two-by-two table for consumption of berries was performed (a cohort study). A chi-squared test was used to calculate the statistical significance.

Most cases (45/46, 98%) had eaten berries. The epidemic curve shows a point-source pattern with some secondary cases (Figure). The incubation period was determined at 32.5 h (range 14-76) and the mean duration of symptoms was 22.4 h (range 1-72 ). Based on

**Table 1**

**Symptoms among cases daycare centre, norovirus outbreak, Finland, October 2009 (n=46)**

| Symptoms | N (%) |
|---|---|
| Vomiting | 42(91) |
| Nausea | 33 (72) |
| Stomach pain | 30 (65) |
| Diarrhoea | 17 (37) |
| Fever | 12 (26) |
| Headache | 10 (22) |

a cohort study, those who ate berries were 3.0 times (relative risk) more likely to develop disease than those who did not ($p \leq 0,05$).

**Food and patient samples from three outbreak settings**

Three samples of frozen raspberries obtained from three outbreak settings (Table 2) and two samples from the wholesaler's stock (total batch size 20,000 kg) were analysed for norovirus at the laboratory of the Department of Virology, Helsinki University Central Hospital (HUSLAB), Helsinki, Finland. The raspberries, packed in bags of 2.5 kg, originated from Poland. Patient samples from two outbreaks were sent for norovirus analysis to HUSLAB and sequencing and genotyping was performed at the laboratory of the Finnish Institute of Health and Welfare. A norovirus real-time RT-PCR was performed targeting the polymerase-capsid gene junction [7]. The sequence analysis was performed on the polymerase and capsid region with primer-pairs MJV12-RegA and SKF-1-SRI-3, respectively [8-10]. The expected lengths of amplicons were 320 and 240bp, respectively. The accession numbers for the norovirus sequences are GU188278 (capsid; berries) and GU 188279 and GU188280 (capsid and polymerase; patient).

**Figure**

**Epidemic curve, norovirus outbreak, day-care center, Finland, October 2009 (n=46)**

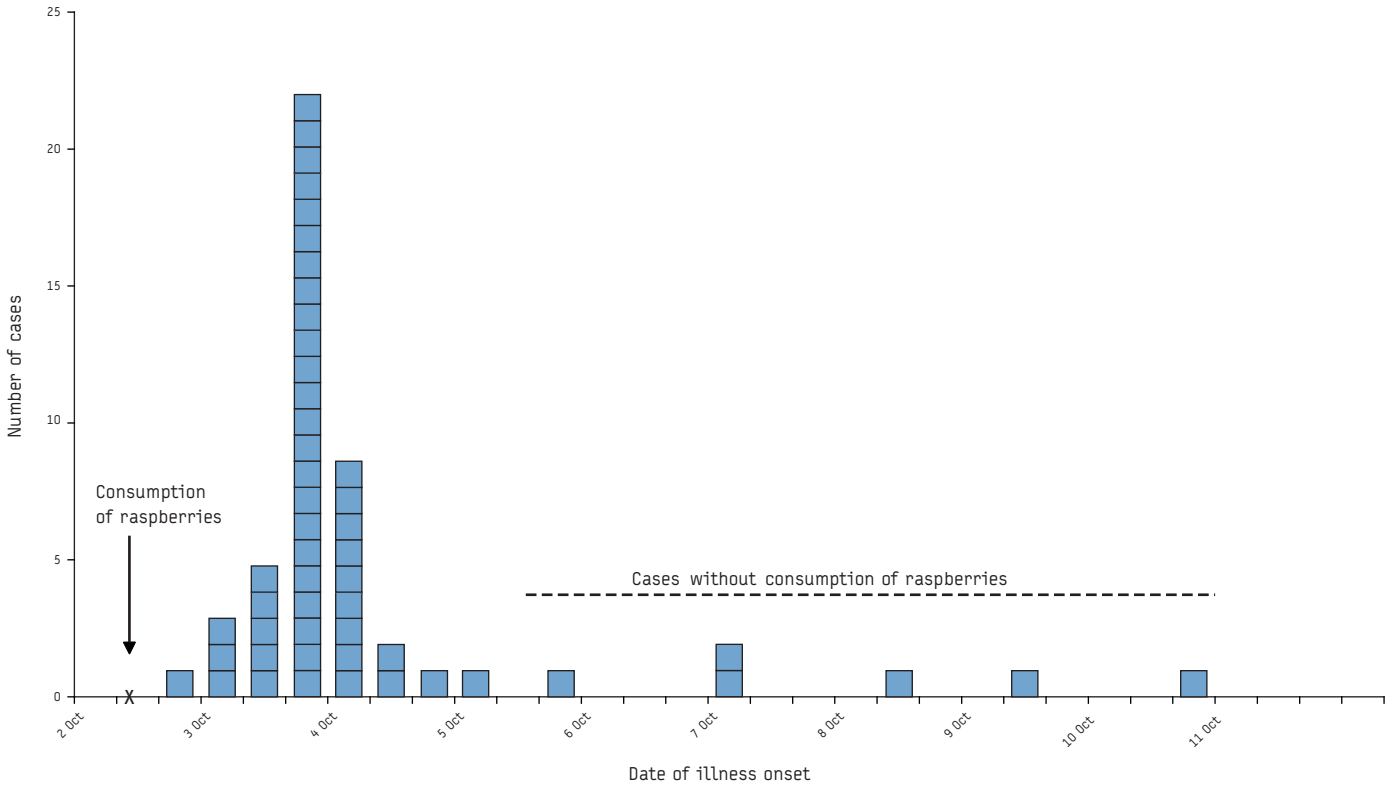


**Table 2**

**Onset of outbreak, number of cases among exposed and detected norovirus, by place, Finland, September-October 2009**

| Place (provider of food) | Start date | Cases/exposed (estimation) | Virus in raspberries | Virus in patients |
|---|---|---|---|---|
| Restaurant (catering) | 26 September | (15/30) | **1/1 NoV GI.4 (Cp 34,8)** | 0/0 |
| **Daycare centre (prepared on site)** | **2 October** | **46/90** | **1/1 NoV GI (Cp 40,1 1:10 37,0)** | **2/3 NoV GI.4** |
| Cafeteria (catering) | 3 October | (15/30) | 1/1 NoV GI (Cp 37,8) | 1/2 NoV |
| Raspberries from wholesaler's stock | NA | NA | 0/2 | NA |

Cp = crossing point -value in norovirus real-time RT-PCR (LightCycler, Roche); NoV: norovirus; NA: not applicable.

### Microbiological findings

In total, norovirus GI was detected in three of five raspberry samples analysed by norovirus real-time RT-PCR. In one case the concentration of virus was high enough to allow exact genotyping and the virus could be identified GI.4 by conventional RT-PCR and sequence determination. In the two patient samples available for genotyping, norovirus GI.4 was detected. The viruses in berries and patients showed identical nucleotide sequences in the short 181 bp-capsid gene region. A positive polymerase RT-PCR result could only be amplified from patient samples.

In addition to the three outbreaks described, several smaller outbreaks involving only few cases (e.g. bakery, bank) that were most likely berry-related, were reported to the local food health authorities between 26 September and 9 October, but no samples were obtained for virological investigation. Taken together, about 200 people were affected in all these outbreaks.

## Discussion and conclusions

Strong laboratory evidence supported the epidemiological findings that imported raspberries were the source of the norovirus outbreaks, since the identical genotype was detected in samples from berries and patients. The outbreaks occurred outside of the norovirus outbreak season that usually occurs from December to May in Finland. The detection of GI.4 virus is in line with a large study of norovirus outbreaks in which the proportion of non-GII.4 outbreaks was found to be higher in food-borne outbreaks, whereas GII.4 outbreaks were mostly linked to person-to-person transmission [11].

The berries that caused the outbreaks were likely to contain a considerable number of viruses, since they were detected without prior concentration of the samples. While the present real-time RT-PCR method is quite sensitive, the positivity in foods is mostly weak, partly due to PCR inhibitors. To determine the viral genotype with the less sensitive conventional RT-PCR is therefore challenging. In this study, a short sequence in a capsid gene region, not normally used for genotyping could be determined in berries, independently from the patient sample analysis.

Our findings highlight the importance of routine investigations of food samples for viral pathogens in addition to bacterial analyses. So far, all our virus findings in foods have been directly linked to outbreaks. In spite of analysing several samples of the same batch of raspberries epidemiologically linked to human cases, norovirus could not be detected in all samples. This could be due to an uneven distribution of viruses in the berries.

The norovirus gastroenteritis outbreaks rapidly died out, after the contaminated batch was withdrawn from the market. Furthermore, the Finnish authorities issued an alert through the Rapid Alert System for Food and Feed (RASFF) on 20 October to inform other European Union countries of the outbreaks caused by norovirus-contaminated raspberries. It is noteworthy that a month earlier, in August, another food-borne outbreak in east Finland was epidemiologically linked to crushed frozen raspberries also imported from Poland. No viruses were found in the berries, but genotype GI.4 norovirus was found in the patients.

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# Single-nucleotide polymorphism in the SCCmec-orfX junction distinguishes between livestock-associated MRSA CC398 and human epidemic MRSA strains

**U Reischl (udo.reischl@klinik.uni-regensburg.de)[1], J Frick[2,3], S Hoermansdorfer[2], H Melzl[1], M Bollwein[1], H J Linde[1], K Becker[4], R Köck[4,5], C Tuschak[2], U Busch[2], A Sing[2]**

1. Institute of Medical Microbiology and Hygiene, University Hospital of Regensburg, Regensburg, Germany
2. Bavarian Health and Food Safety Authority, Oberschleissheim, Germany
3. Clinic for Swine, Veterinary Faculty, Ludwig-Maximilians-University, Oberschleissheim, Germany
4. Institute of Medical Microbiology, University Hospital Münster, Münster, Germany
5. Institute of Hygiene, University Hospital Münster, Münster, Germany

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A number of real-time PCR assays for direct detection of methicillin-resistant (MRSA) in clinical specimens are targeting staphylococcal cassette chromosome mec (SCCmec) right extremity sequences and the *S. aureus* chromosomal *orfX* gene sequences located to the right of the SCC*mec* integration site. When testing 184 MRSA strains of human and animal origin from geographically distinct locations, we identified several characteristic single-nucleotide polymorphisms (SNPs) within the SCC*mec-orfX* junction of livestock-associated (LA) MRSA CC398 which serve as suitable strain markers for screening purposes. Within an assay time of 60 minutes and an additional 10 minutes for the melting curve analysis, all MRSA CC398 isolates were correctly identified by their characteristic $T_m$ value in the commercial LightCycler MRSA Advanced test. Studies to confirm the diagnostic accuracy of the SNP-based strain identification assay with a larger collection of clinical and LA-MRSA strains are ongoing.

## Introduction

Rates of methicillin-resistant *Staphylococcus aureus* (MRSA) infections have steadily increased during the past two decades and the occurrence and spread of MRSA strains in healthcare facilities as well as in the community is a growing problem worldwide [1,2,3]. Although classically considered as a nosocomial pathogen, reports of MRSA carriage or its acquisition in the community have become increasingly common during the past decade [2,4]. More recently, studies have demonstrated that especially swine and swine farmers in Austria [5], Denmark [6], Germany [7,8], the Netherlands [9-11], Portugal [12], the United States [13], and many other countries are colonised with MRSA. Since it was realised that livestock animals may form a new, separate reservoir, these strains are now called livestock-associated MRSA (LA-MRSA). Molecular characterisation revealed that a distinct clone of MRSA is predominant within this collective: LA-MRSA strains are grouping within the new clonal complex 398 (CC398) with sequence type 398 (ST398) as the most prevalent type. Animals carrying MRSA represent a reservoir for transmission to humans [13,14,15]. The MRSA strains from animal origin have been shown to be pathogenic for humans and can cause severe infections such as endocarditis and ventilator-associated pneumonia [16]. A number of diagnostic strategies have been published on the molecular characterisation of the MRSA CC398 clonal lineage, using pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) or other laborious techniques based on genome sequencing [1,19,20].

### Active surveillance is needed

Since livestock animals may act as a reservoir for this bacterium, the development of rapid molecular methods for screening and identification of MRSA CC398 will have important implications for surveillance, epidemiology and future infection control initiatives. As with many other bacterial pathogens, the rapid and reliable detection of certain MRSA clones is of the utmost importance to prevent the spread of infection. A number of real-time PCR assays targeting staphylococcal cassette chromosome *mec* (SCC*mec*) right extremity sequences and the *S. aureus* chromosomal *orfX* gene sequences located to the right of the SCC*mec* integration site have recently been established for direct detection of MRSA in clinical specimens. In the course of the present study, such assays were evaluated for their performance to detect and distinguish LA-MRSA strains of the new clonal complex 398 (CC398).

## Materials and Methods

### Study population, survey methods and diversity of investigated strains

During an on-going study conducted by the Bavarian Health and Food Safety Authority to explore the epidemiology of MRSA CC398 in Bavaria, the MRSA colonisation status among swine bred in Bavaria and the involved farmers was investigated by sampling the nares of 634 swine and 116 farmers on 60 geographically distinct farms. Epidemiological results will be available when this particular study is completed. Additional representatives of the MRSA CC398 lineage from other geographic locations and other sources including horses, dogs, guinea pigs, chicken, poultry and humans with contact to colonised animals, as well as MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates of presumed to be related spa-types were kindly provided by a number of supporting laboratories listed in the Table and in the Acknowledgements section.

**Diagnostic culture and template DNA preparation**

The *S. aureus* strains collected from pigs and farmers in Bavaria were maintained on Columbia blood agar and identified by colony morphology, Gram-stain characteristics, catalase reaction, coagulase production, and the results of the API Staph system (bioMérieux). Oxacillin susceptibility was determined by the agar screening method with Mueller-Hinton agar containing 2% NaCl and 6 mg/l of oxacillin for *S. aureus* [3,19]. An identical protocol was applied for characterisation and maintaining the various *S. aureus* strains provided by supporting laboratories. Template DNA for PCR was prepared from individual bacterial colonies by a simple and rapid 'boiling' procedure [20]. Briefly, colonies were suspended in 200 µl of lysis buffer containing 1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA, and incubated for 10 min at boiling temperature. After centrifugation for 2 min at 10.000 x g to sediment the debris, a 2 µl aliquot of the clear supernatant was directly transferred to PCR. Alternatively, the reagents and the protocol of the LightCycler Advanced Lysis kit (Roche Diagnostics) were used for template DNA preparation. For cultured bacterial organisms, the efficiencies of the commercial lysis kit and the 'boiling' procedure were found to be comparable for the extraction of amplifiable *S. aureus* DNA (data not shown).

**PCR amplification, DNA sequencing and molecular strain typing**

Amplification and sequencing of the SCC*mec-orfX* junction was performed according to Hagen et al. [21]. PCR reactions and subsequent hybridisation probe melting curve analyses were carried out on a Roche LightCylcer 2.0 device. Amplicons of the expected size were purified (HighPure PCR Cleanup Micro kit, Roche Diagnostics) and sequenced on an automated ABI 310 sequencer using BigDye v. 1.1 chemistry.

Real-time PCR amplification and detection reactions were carried out according to the protocol of the LightCycler MRSA Advanced test (Roche Diagnostics). In the case of a negative result, an in-house duplex PCR assay was performed targeting a segment of the *mec*A gene and the S. aureus-specific genomic fragment Sa 442 [22]. For selected *S. aureus* strains, accessory testing was performed with well-established commercial PCR tests designed for direct detection of MRSA from clinical specimens, namely the GenoType MRSA Direct (Hain Lifescience), the BD GeneOhm MRSA (Becton Dickinson) and the Xpert MRSA (Cepheid) assays.

The presence of Panton-Valentine leukocidin (PVL) [23] was investigated by PCR testing for the lukS-PVand lukF-PV genes [2,4]. Typing of the *S. aureus* protein A gene (spa) was performed for all isolates obtained in this study using a standard protocol [24]. Clustering of spa types into spa clonal complexes (spa-CC) was performed using the Based Upon Repeat Pattern (BURP) algorithm of the Ridom StaphType software (Ridom GmbH) with the following preset parameters as recommended previously [25]: Spa types were clustered into the same group if the cost was four or less; spa types which were shorter than five repeats were excluded. When an isolate was indicated to be closely related to a spa type presumed to be associated with CC398, but the $T_m$ values observed in the LightCycler MRSA Advanced test did not correspond to the $T_m$ values expected for CC398 isolates, MLST- and SCC*mec*-types of the isolates were determined [1] or provided by the supporting

**Table**

**MRSA isolates investigated in the present study (n=184)**

| Number of tested strains | MLST | *spa* type | $T_m$ value (°C) | *mecA* | Sa 422 | Xpert MRSA | BD GeneOhm MRSA | Source | Geographic origin | Culture result | Comment |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 8 | ST 398 | t011 | 55 | n.d. | n.d. | pos. | pos. | Piglets | Germany (center 1) | MRSA | Bavarian LA-MRSA survey |
| 11 | ST 398 | t034 | 55 | n.d. | n.d. | pos. | pos. | Piglets | Germany (center 1) | MRSA | Bavarian LA-MRSA survey |
| 11 | ST 398 | t011 | 55 | n.d. | n.d. | pos. | pos. | Fattening pigs | Germany (center 1) | MRSA | Bavarian LA-MRSA survey |
| 5 | ST 398 | t034 | 55 | n.d. | n.d. | pos. | pos. | Fattening pigs | Germany (center 1) | MRSA | Bavarian LA-MRSA survey |
| 5 | ST 398 | t011 | 55 | n.d. | n.d. | pos. | pos. | Humans (pig farmers) | Germany (center 1) | MRSA | Bavarian LA-MRSA survey |
| 4 | ST 398 | t034 | 55 | n.d. | n.d. | pos. | pos. | Humans (pig farmers) | Germany (center 1) | MRSA | Bavarian LA-MRSA survey |
| 1 | ST 398 | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Pig | Germany (center 2) | MRSA | |
| 1 | ST 398 | t2510 | 55 | n.d. | n.d. | n.d. | n.d. | Pig | Germany (center 2) | MRSA | |
| 1 | ST 398 | t1451 | 55 | n.d. | n.d. | n.d. | n.d. | Pig | Germany (center 2) | MRSA | |
| 1 | ST 398 | t108 | 55 | n.d. | n.d. | n.d. | n.d. | Pig | Germany (center 2) | MRSA | |
| 13 | ST 398 | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Human (pig farmers conference) | Austria (center 3) | MRSA | SCCmec type V |
| 1 | ST 398 | t2576 | 55 | n.d. | n.d. | n.d. | n.d. | Human (veterinarian conference) | Austria, Germany, Switzerland (center 3) | MRSA | SCCmec type V |
| 7 | ST 398 | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Human (veterinarian conference) | Austria, Germany, Switzerland (center 3) | MRSA | SCCmec type V |
| 3 | ST 398 | t034 | 55 | n.d. | n.d. | n.d. | n.d. | Human (veterinarian conference) | Austria, Germany, Switzerland (center 3) | MRSA | SCCmec type V |
| 13 | ST 398 | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Human (pig farmers and veterinarians) | Austria (center 3) | MRSA | SCCmec type V |
| 24 | ST 398 | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Dust (from pig breeding facilities) | Austria (center 3) | MRSA | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ST 398 | t034 | 55 | n.d. | n.d. | n.d. | n.d. | Dust (from pig breeding facility) | Austria (center 3) | MRSA | |
| 10 | ST 398 | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Pigs | Germany (center 8) | MRSA | |
| 1 | ST 398 | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Human (wound swab) | Germany (center 1) | MRSA | |
| 1 | ST 398 | t1456 | 55 | n.d. | n.d. | n.d. | n.d. | Human (wound swab) | Germany (center 1) | MRSA | |
| 1 | ST 398 | t1456 | 55 | n.d. | n.d. | n.d. | n.d. | Human (wound swab) | Germany (center 1) | MRSA | |
| 1 | ST 398 | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 5) | MRSA | |
| 7 | ST 398 | t108 | 55 | n.d. | n.d. | n.d. | n.d. | Humans | Netherlands (center 7) | MRSA | |
| 2 | ST 398 | t034 | 55 | n.d. | n.d. | n.d. | n.d. | Humans | Netherlands (center 7) | MRSA | |
| 9 | ST 398 | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Humans | Netherlands (center 7) | MRSA | |
| 2 | ST 398 | t034 | 55 | n.d. | n.d. | n.d. | n.d. | Humans | Denmark (center 6) | MRSA | SCCmec type V |
| 1 | ST 398 | t108 | 55 | n.d. | n.d. | n.d. | n.d. | Human | Denmark (center 6) | MRSA | SCCmec type V |
| 2 | ST 398 | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Humans | Denmark (center 6) | MRSA | |
| 1 | ST 398 | t5706 | 55 | n.d. | n.d. | n.d. | n.d. | Human | Denmark (center 6) | MRSA | |
| 1 | ST 398 | t108 | 55 | n.d. | n.d. | n.d. | n.d. | Human | Denmark (center 6) | MRSA | PVL positive, SCCmec type V |
| 1 | n.d. | t1793 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | PVL positive |
| 1 | n.d. | t1250 | 55 | n.d. | n.d. | n.d. | n.d. | Pig | Germany (center 2) | MRSA | |
| 2 | n.d. | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Poultry | Germany (center 2) | MRSA | |
| 1 | n.d. | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Guinea pig | Germany (center 2) | MRSA | |
| 1 | n.d. | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Dog | Germany (center 2) | MRSA | |
| 3 | ST 398 | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Horses | Germany (center 2) | MRSA | |
| 1 | n.d. | t1457 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t1580 | 55 | n.d. | n.d. | n.d. | n.d. | Human (pharynx) | Germany (center 2) | MRSA | |
| 1 | n.d. | t2011 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t1451 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t2346 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t2370 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t2576 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t2741 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t3423 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t1255 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t1197 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t571 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t108 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t2582 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | n.d. | t034 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | ST 398 | t011 | 55 | n.d. | n.d. | n.d. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | ST 30 | t138 | 59 | pos. | pos. | pos. | n.d. | Human (nose) | Germany (center 2) | MRSA | |
| 1 | ST 9 | t1430 | 59 | pos. | pos. | pos. | n.d. | Chicken (wing) | Germany (center 4) | MRSA | SCCmec type IVa |
| 1 | ST 1 | t127 | 59 | pos. | pos. | pos. | pos. | Piglet (nose) | Germany (center 1) | MRSA | |
| 1 | ST 398 | t034 | neg. | pos. | pos. | neg. | neg. | Human | Denmark (center 6) | MRSA | SCCmec type IVa |
| 1 | ST 398 | t034 | neg. | pos. | pos. | neg. | neg. | Human | Denmark (center 6) | MRSA | SCCmec type VII |
| 1 | ST 398 | t571 | neg. | pos. | pos. | neg. | neg. | Human | Denmark (center 6) | MRSA | PVL positive, SCCmec non-typeable |
| 1 | ST 398 | t1606 | neg. | pos. | pos. | neg. | neg. | Human (nose) | Germany (center 2) | MRSA | SCCmec non-typeable |
| 1 | ST 753 | t898 | neg. | pos. | pos. | neg. | neg. | Human (nose) | Germany (center 2) | MRSA | SCCmec non-typeable |
| 1 | ST 398 | t567 | neg. | pos. | pos. | neg. | neg. | Human (nose) | Germany (center 2) | MRSA | SCCmec non-typeable |
| 1 | ST 30 | t021 | neg. | pos. | pos. | neg. | neg. | Human (nose) | Germany (center 2) | MRSA | SCCmec non-typeable |

BD GeneOhm MRSA: test result of a commercial MRSA-specific PCR assay (Becton Dickinson); MLST: multilocus sequence typing; MRSA: methicillin-resistant *Staphylococcus aureus*; *mecA*: test result of an in-house realtime PCR assay targeting the *mecA* gene [20]; neg: no specific amplification products observed or negative test result for MRSA; n.d. not done; pos: specific amplification products observed or positive test result with the applied realtime PCR assays designed for direct detection of MRSA by targeting the SCC*mec-orfX* integration site; pSA422: test result of an in-house realtime PCR assay targeting a *S. aureus*-specific species marker gene Sa422 [20];$T_m$: $T_m$-value observed with the LightCycler MRSA Advanced Test (Roche Diagnostics); Xpert MRSA: test result of a commercial MRSA-specific PCR assay (Cepheid).

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**Figure 1**

**Multiple sequence alignment of a selected *S. aureus orfX* segment**

```
FJ830606  CCGCATCATTTGGTGTGGAAATGTCATTTTGCTGAATGATA porcine MLST 398
AB033763  ............A.....G..................... SCCmec Type I
D86934    ............A.....G..................... SCCmec Type II
AB047089  ............A.....G..................... SCCmec Type III
AB063172  ............A.....G.............A....... SCCmec Type IVa
AB063173  ............A.....G..................... SCCmec Type IVb
AB096217  ............A.....G..................... SCCmec Type IVc
DQ106887  ............A.....G..................... SCCmec Type IVg
AB121219  ............A.....G.............A....... SCCmec Type V
AF411935  ............A.....G..................... SCCmec Type VI
AB373032  ............A.....G..................... SCCmec Type VII
FJ390057  ............A.....G..................... SCCmec Type VIII
AM292304  ............A.....G..................... SCCmec Type unknown
AB425823  ............A........................... SCCmec Type IVa
U10927    ..T.................................... SCCmec Type unknown
          |           |     |           |         |
Pos.      300         312   318         330       340  in FJ830606

FJ830606  GTGCGTAGTTACTGCGTTGTAAGACATCCTTGTGCAGGCCG porcine MLST 398
AB033763  ........................G.............. SCCmec Type I
D86934    ........................G.............. SCCmec Type II
AB047089  ........................G.............. SCCmec Type III
AB063172  ....A...................G.............. SCCmec Type IVa
AB063173  ........................G.............. SCCmec Type IVb
AB096217  ........................G.............. SCCmec Type IVc
DQ106887  ........................G.............. SCCmec Type IVg
AB121219  ....A...................G.............. SCCmec Type V
AF411935  ........................G.............. SCCmec Type VI
AB373032  ........................G.............. SCCmec Type VII
FJ390057  ........................G.............. SCCmec Type VIII
AM292304  ........................G.............. SCCmec Type unknown
AB425823  ............A...........G.............. SCCmec Type IVa
U10927    ........................G.............. SCCmec Type unknown
          |         |         |     |             |
Pos.      341       350       360   366           381 in FJ830606

FJ830606  TTTGATCCGCCAATGACGAATACAAAGTCGCTTTGCCCTTG porcine MLST 398
AB033763  ..................A..................... SCCmec Type I
D86934    ........................................ SCCmec Type II
AB047089  ........................................ SCCmec Type III
AB063172  ........................................ SCCmec Type IVa
AB063173  ..................A..................... SCCmec Type IVb
AB096217  ........................................ SCCmec Type IVc
DQ106887  ........................................ SCCmec Type IVg
AB121219  ........................................ SCCmec Type V
AF411935  ........................................ SCCmec Type VI
AB373032  ........................................ SCCmec Type VII
FJ390057  ........................................ SCCmec Type VIII
AM292304  ........................................ SCCmec Type unknown
AB425823  ........................................ SCCmec Type IVa
U10927    ........................................ SCCmec Type unknown
          |         |         |         |           |
Pos.      382       390       400       410         422 in FJ830606
```

Multiple sequence alignment of a selected *S. aureus orfX* segment located close to the SCC*mec-orfX* junction (position 253 in GenBank FJ830606). The most similar sequences found in BLAST search show either a sequence identical to GenBank sequence entries of *S. aureus* isolates carrying one of the eight *SCCmec* types or differ from the MRSA ST 398 isolates of the study by at least two nucleotides at positions 312 and 366 (GenBank FJ830606).

laboratories. Typing of SCC*mec* elements of types I to VII was carried out according to previously published PCR procedures [26].

## Results

### Molecular characteristics of MRSA isolates derived from the Bavarian LA-MRSA survey

By sampling the nares of 634 swine and 116 farmers on 60 geographically distinct farms in Bavaria during the course of an ongoing study, a total number of 245 MRSA strains from pigs and 34 MRSA strains from farmers were grown from the collected swabs. From this collection, 44 MRSA isolates from geographically distinct farms were chosen for further analyses (Table, rows 1 to 6). The distribution of spa types among these isolates was as follows: t011 (n=24) and t034 (n=20). MLST-typing of all selected MRSA isolates revealed that they belonged to MLST ST398. All 44 MRSA strains tested negative for PVL-encoding genes.

### Novel single nucleotide polymorphisms in the SCC*mec-orfX* integration site of LA-MRSA isolates

By systematic sequencing of the SCC*mec-orfX* integration sites of MRSA isolates of animal origin, all of the 44 sequences obtained from Bavarian porcine isolates (Table, rows 1 to 6) were found to be identical in a multiple alignment (using pileup from the HUSAR sequence analysis package from the German Cancer Research Center (DKFZ), http://genius.embnet.dkfz-heidelberg.de, data not shown). As the sequence differed from previously published SCC*mec-orfX* integration site sequence motifs, it was deposited in GenBank with accession number FJ830606 (to be released after publication).

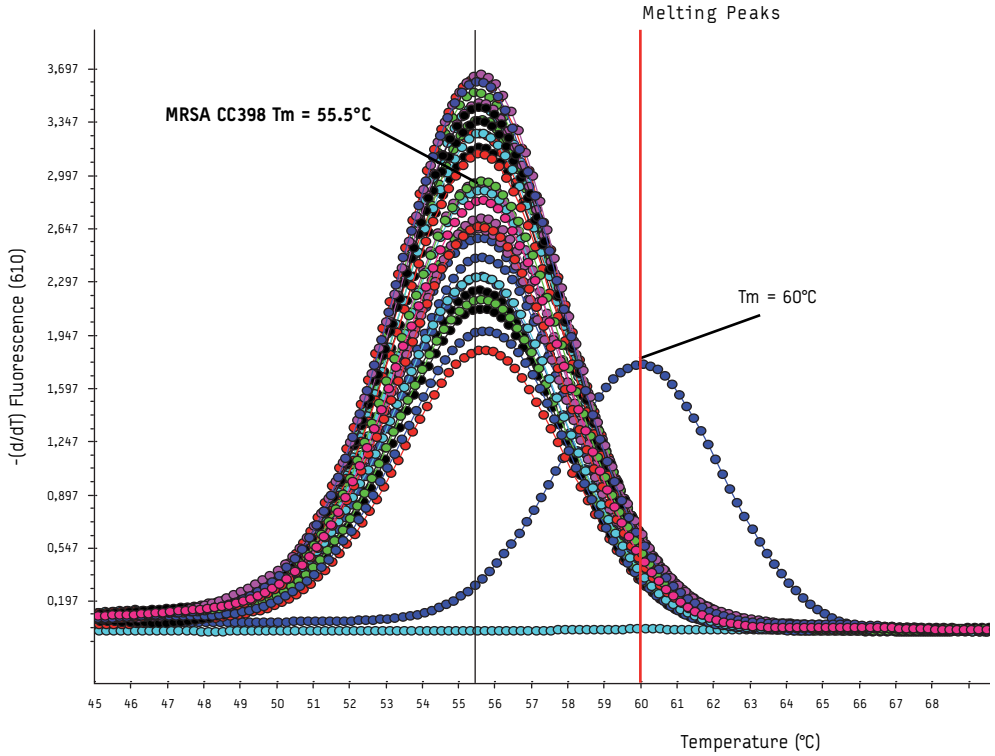
A detailed investigation of the novel sequence revealed some nucleotide positions uncommon in *S. aureus* GenBank sequences and at least three characteristic single nucleotide polymorphisms (SNPs) in the *S. aureus* chromosomal *orfX* gene sequence located to the right of the SCC*mec* integration site: guanine at position 312, adenine at position 366, and cytosine at position 441 (GenBank FJ830606). At least two of these SNPs were found exclusively in the investigated MRSA strains of animal origin and may serve as a diagnostic marker for the presence of MRSA CC398. A BLAST search (National Center for Biotechnology Information (NCBI), http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the complete amplicon sequence revealed GenBank accession number AM292304 (*S. aureus* SCC*mec*ZH47 mobile element) as the most similar hit with five mismatches. GenBank AB425823 and U10927, the next similar sequences found in the BLAST search, were either identical to GenBank entries of one of the eight acknowledged MRSA SCC*mec* types deposited in GenBank, or had at least one nucleotide difference at position 366 compared with the sequence FJ830606 obtained from the investigated MRSA ST398 strains of porcine origin (Figure 1).

### Practical application of the identified single nucleotide polymorphisms

In addition to the broad spectrum of unpublished in-house PCR protocols, also the proprietary sensor hybridisation probe of

**Figure 2**

**Specificity of the Roche LightCycler MRSA Advanced test for differentiating MRSA CC398 and non-CC398 strains in hybridisation probe melting curve analysis**




The curves represent the MRSA strain ATCC 33592 ($T_m \approx 60$ °C) and 30 MRSA ST 398 strains of porcine origin with characteristic Tm values around 55.5 °C. A methicillin-sensitive strain of *S. aureus* (clinical isolate) was used as negative control.

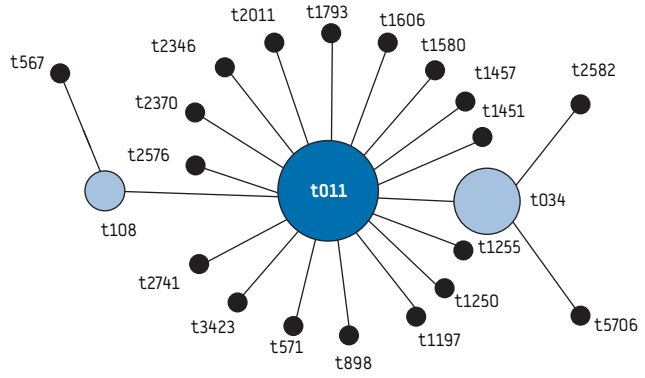
the recently developed LightCycler MRSA Advanced Test (Roche Diagnostics) covers one of these two particular nucleotide positions. This real-time PCR assay detects MRSA strains with different molecular sequences surrounding the right extremity junction of the SCC*mec* cassette with the *S. aureus orfX* gene. As a practical application of the SNPs identified in our study, we present the use of this commercial real-time PCR kit for the direct detection of MRSA and simultaneous identification of LA-MRSA CC398. For all 44 investigated Bavarian MRSA ST398 strains, specific amplification products were generated with the LightCycler MRSA Advanced test, and they all harboured at least one of the identified SNPs in the SCC*mec-orfX* junction represented by a characteristic $T_m$ of 55.5 °C in the subsequent LightCycler hybridisation probe melting curve analysis (Figure 2). Since we have not yet observed such a Tm-shift with any non-ST398 MRSA strains of human or animal origin, this point mutation may serve as a molecular marker for the presence of MRSA CC398.

As an approved in vitro diagnostics (IVD) product, the Roche LightCycler MRSA Advanced test has already been validated with a comprehensive collection of MRSA strains of human origin for the limit of detection, inclusivity and exclusivity. The results of systematic studies on the assay's diagnostic performance will be published soon (personal communication, Roche Diagnostics). According to the product information of the test kit, the range of $T_m$ values observed in these multicenter validation studies with various epidemic MRSA clones of human origin was from 57.0 to 62.0 °C. Therefore a $T_m$ value of 55.5 °C observed with MRSA CC398 should be discriminative with respect to most of the clinical MRSA strains, and melting curve analysis represents a reliable surrogate marker for screening purposes.

From a technical point of view, it should be noted that melting points outside the expected range of 57.0 to 62.0 °C have to be examined manually in the LightCycler software. When testing MRSA CC398 strains of the present study, the calculation algorithms embedded in the automated assay interpretation software of the LightCycler MRSA Advanced test (Micro Analysis Software; MAS) reported "MRSA result: not detected" with a specific comment "Peak(s) outside Target TM range".

**Figure 3**

**Population snapshot of the tested isolates based on BURP analysis (n=127)**




Each dot represents a single *spa* type and the diameter of the dot reflects the number of isolates associated with the respective *spa* type. Group founders are coloured in blue, Subgroup founders are coloured in light blue.

In the course of the study, we also applied a number of other commercial PCR tests targeting the SCC*mec-orfX* junction. These included the GenoType MRSA Direct (results not shown), the BD GeneOhm MRSA, and the Xpert MRSA test. The 44 investigated Bavarian MLST CC398 strains, which had all tested positive in the LightCycler MRSA Advanced test, also tested positive for MRSA in these other assays (see Table) - but these PCR test platforms either did not have an option to perform a hybridization probe melting curve analysis or did not allowviewing such melting curve data. Since clinical sensitivity of real-time PCR assays may also depend on the annealing temperatures of the respective probes, it is currently unclear whether the point mutations in the target region will have an impact on the sensitivity when testing samples from patients or animals.

### Testing of non-Bavarian MRSA strains within or related to MLST CC398

In addition to the 44 strains of the Bavarian porcine LA-MRSA survey, 140 MRSA strains recovered from animals and humans in other geographical regions or from other animal sources as well as *S. aureus* isolates of spa-types sharing similar spa repeat patterns, were included in the present study to further address the diversity among isolates within the MLST CC398 clonal complex. Overall, 133 of the 140 isolates were successfully detected by the LightCycler MRSA Advanced test. The collection of investigated strains is shown in detail in the Table, together with the characteristic $T_m$ values observed in the LightCycler MRSA Advanced test and the corresponding results of supplementary S. aureus- and *mec*A-specific PCR assays, as well as the results obtained in other commercial PCR tests targeting the SCC*mec-orfX* junction. While seven isolates were not detectable, 130 isolates were associated with $T_m$ values of 55.5 °C in the LightCycler MRSA Advanced test, indicative of the presence of the novel SNPs, and three isolates were associated with $T_m$ values of 59 °C, known to be within the range observed for the epidemic MRSA clones of human origin.

All of the applied real-time PCR assays, which are designed for direct detection of MRSA by targeting the SCC*mec-orfX* integration site, failed to generate specific amplification products with seven (3.8%) of the investigated MRSA strains (Table). The MRSA phenotype of these strains was confirmed by diagnostic culture including oxacillin susceptibility testing. In addition, the MRSA genotype was confirmed by an in-house duplex PCR assay targeting the *mec*A gene and a S. aureus-specific species marker. SCC*mec* typing of these seven isolates revealed that one was associated with SCC*mec* IVa, one with SCC*mec* VII and five isolates were not typeable using a common SCC*mec* typing approach.

A population snapshot based on the BURP algorithm was performed for all MRSA isolates included in the study (Figure 3). For arithmetical reasons, three isolates characterised by a $T_m$ of 55.5 °C (two t1456 isolates and one t2510 isolate, all typed as MLST ST398) were excluded from spa cluster formation by BURP because they were shorter than five repeats. The snapshot showed that all remaining 127 isolates associated with $T_m$ values of 55.5 °C clustered into one spa-CC. This spa-CC comprised the major spa types t011 and t034 shown to be associated with MLST ST398. This spa-CC contained a further 20 spa types sharing closely related spa repeat patterns: t108, t567, t571, t898, t1197, t1250, t1255, t1451, t1457, t1580, t1606, t1793, t2011, t2346, t2370, t2576, t2582, t2741, t3423 and t5706.

Moreover, six of the seven isolates not detected by the LightCycler MRSA Advanced test clustered in this spa-CC. MLST typing revealed that five isolates (two of spa type t034 and one each of types t567, t571 and t1606) were associated with ST398, and one isolate associated with spa type t898 was MLST ST753 (90-35-19-2-20-26-39), which is closely related to ST398 (3-35-19-2-20-26-39). Thus, all these six isolates were part of the CC398 complex. The remaining isolate not detected by the LightCycler MRSA Advanced test was associated with spa type t021 (ST30).

Those three strains that were characterised by a $T_m$ of 59.0 oC in the LightCycler MRSA Advanced test showed spa types t127 (ST1), t138 (ST30) and t1430 (ST9).

## Discussion and conclusions

Although a number of comprehensive studies have been published on the molecular characterisation and detection of the CC398 clonal MRSA lineage using PFGE, MLST or other techniques based on genome sequencing [1,16], this is the first report on a truly rapid detection and/or screening method for this livestock-associated clonal lineage based on characteristic SNPs within a popular target sequence of MRSA-specific PCR assay.

Here, 184 different LA-MRSA isolates obtained from various geographic regions in several European countries and from different sources including pigs, horses, dogs, guinea pigs, chicken, poultry as well as associated in-contact humans were systematically investigated for a characteristic SNP-induced Tm-shift in the LightCycler MRSA Advanced test.

The novel SNPs within the *S. aureus* chromosomal *orfX* gene detected in the investigated LA-MRSA isolates seemed to represent a conserved sequence motif for these MRSA strains. Even if seven of 184 MRSA strains (six of which were LA-MRSA CC398) were not picked up by the assays due to the presence of uncommon SCC*mec* elements, it can be stated that the investigated commercial PCR tests targeting the SCC*mec-orfX* junction showed acceptable inclusivity rates for members of the MRSA CC398 complex. A spa type population snapshot applying the BURP algorithm showed that all MRSA isolates characterised by the SNP-induced Tm-shift in the LightCycler MRSA Advanced test clustered into a distinct spa clonal complex indicative for CC398. Therefore, the novel SNPs within the *S. aureus* chromosomal *orfX* gene sequences could serve as a discriminative marker for MRSA belonging to the CC398 complex.

It is a well known fact that primer and probe sequences of the current PCR assay concepts are designed to cover the most common SCC*mec* types encountered in clinical MRSA isolates. With our increasing knowledge about the enormous sequence diversity of SCC*mec* sequences, rational primer selection and assay design can only be a best compromise between the coverage of as many SCC*mec* variants as possible and loss of analytical sensitivity due to primer multiplexing problems in the PCR reaction mixture.

In the course of the present study, we identified a powerful additional feature of the commercial Roche LightCycler MRSA Advanced test. This observation is another example for the fact that the natural diversity of MRSA is also reflected on genomic level. The more isolates are tested for a given target sequence, the more nucleotide mutations or deletions may be encountered. This fact has also implications on the design of specificity panels when developing assays. The assay panel covering epidemiologically relevant clones frequently encountered in patients at risk for MRSA infection is not necessarily congruent with the spectrum of variant isolates that may be found in a specific geographical or epidemiological setting (e.g. introduction of LA-MRSA lineages into a hospital setting). A recent study by Bartels et al. [25] highlighted this problem reporting on a variant SCC*mec* type IVa clone (spa t024 ST 8) circulating in Copenhagen, which was not detected by a commercial real-time PCR assay targeting the SCC*mec-orfX* junction.

Now that characteristic SNPs have been identified, colleagues may verify our findings with their collections of animal-associated MRSA strains and may check the primer and probe sequences of their individual in-house PCR protocols targeting the SCC*mec-orfX* junction for the ability to cover and/or to discriminate MRSA CC398 from human MRSA clones.

If the LightCycler MRSA Advanced test was implemented in a diagnostic laboratory for the intended purpose of direct detection of MRSA in clinical specimens, the occurrence of presumptive MRSA CC398 strains could be monitored without extra work or extra cost just by looking at the melting curve screen. In combination with the simple 'boiling'-protocol for template DNA preparation, it can be easily integrated into the workflow of any clinical or veterinary laboratory routinely using molecular techniques for diagnostic purposes. Once growth of staphylococci is observed on agar plates, a portion of the colony can be transferred to PCR and discriminative MRSA results can be available within 80 minutes. Moreover, knowing about our study results, users of this assay will no longer be confused by the comment "Peak(s) outside Target TM range" generated by the automated assay interpretation software.

In conclusion, the characteristic SNP-induced Tm-shift found in the LightCycler MRSA Advanced test was shown to be suitable to rapidly identify LA-MRSA CC398 clones. By simultaneous screening for general MRSA carriage as well as for MRSA CC398 carriage, this commercial real-time PCR test or comparable assay designs may help to monitor the spread of MRSA CC398 in the human population and, in particular, its importation into healthcare settings. Moreover, this approach may be helpful in screening for MRSA CC398 carriage among animals, farmers or other risk groups.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# Viral hepatitis, HIV, human herpes virus and Treponema pallidum infection in haemodialysis patients from Kosovo, 2005

**G L Quaglio (gianluca.quaglio@azosp.vr.it)[1], C Pattaro[2], N Ramadani[3], L Bertinato[1], Y Elezi[4], P Dentico[5], A Volpe[5], M Ciotti[6], G Rezza[7], G Putoto[1]**

1. Veneto Region, Italian Co-operation, Peja Training Project Team, Venice, Italy
2. European Academy (EURAC), Bozen/Bolzano, Italy - Affiliated Institute of the University Lübeck, Germany
3. National Institute of Public Health, Pristhine, Kosovo
4. Nephrology Unit, Department of Internal Medicine, University of Prishtine, Kosovo
5. Institute of Internal Medicine, University of Bari, Italy
6. European Centre for Disease Prevention and Control, Stockholm, Sweden
7. Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy

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The serological status of hepatitis viruses and other infectious diseases in the 66 dialysed patients of one haemodialysis unit in Kosovo were studied, comparing the data with a large group of blood donors and out-patients. All dialysed patients were hepatitis A virus (HAV) positive. Prevalence of hepatitis B surface antigen (HBsAg), hepatitis B surface antibodies (anti-HBs), and hepatitis B core antibodies (anti-HBc) was 14 of 66, 21% (95% confidence interval (CI): 12-33%), 5 of 66, 8% (95%CI: 5-22%), and 50 of 66, 76% (95%CI: 64-85%), respectively. Antibodies to hepatitis C virus (anti-HCV) prevalence was 57 of 66, 86% (95%CI: 76-94%). No human immunodeficiency virus (HIV) positive case was found. Prevalence of past herpes simplex virus type 2 (HSV-2) infection was 29% (95%CI: 18-41%). Two patients (3%, 95%CI: 0-10%) were positive for *Treponema pallidum* and 18% (95%CI: 10-30%) were human herpesvirus 8 (HHV-8) antibody positive. Four hundred and fifty-two subjects were recruited for comparison. Markers of past HAV infection was associated with haemodialysis (Fisher's exact test p-value=0.037). Dialysed patients were at a higher risk of being HBsAg positive than others: the sex- and age-adjusted odds ratio (OR) was 5.18 (95%CI: 1.87-14.32). Anti-HBc positivity was strongly associated with haemodialysis: the sex- and age-adjusted OR was 6.43 (95%CI: 3.22-12-85). Anti-HCV positivity was 86% and 1% in presence and absence of haemodialysis, respectively. The Fisher's exact test for association proved a strong association between haemodialysis and HCV (p-value<0.0001). The OR for association between haemodialysis and HSV-2 positivity was 3.20 (95%CI: 1.46-7.00). Significant associations were also observed between haemodialysis status and antibodies to *Treponema pallidum* (Fisher's exact test p-value=0.044). In Kosovo, the prevalence of viral hepatitis infection and other viral infections and *Treponema pallidum* among dialysed patients is high, indicating major ongoing nosocomial transmission.

## Introduction

The population of Kosovo has suffered substantially after the break-up of the former Yugoslavia in the early 1990ies and the consequent armed conflict in 1999. Recently, the region has acquired a national autonomy, with some limitations of sovereignty and with the support of the European Union [1]. In 2006, the population was estimated at 1.9 million and was one of the youngest in Europe. About 37% lived in poverty; unemployment was estimated at around 40%, with a gross domestic product per capita of 834 EUR in 2006 (468 EUR in 2000) [2]. Health indicators remained among the most unfavourable in the Balkan region. The annual per capita government expenditure on health care was 35 EUR, the lowest in Europe. Kosovo had one of the highest perinatal mortality rates (23 per 1,000 live births) in Europe and the number of physicians per 1,000 inhabitants was 0.94 [3]. The transition to more modern concepts of health care management presented a challenge to health personnel and the population after the war. Currently, the healthcare system consists of primary centers located in each municipality, secondary health care facilities at the regional level (five hospitals), and tertiary health care centers (University of Pristine and a few other specialised institutions).

After the conflict, the number of end-stage renal disease (ESRD) patients progressively increased in Kosovo: from 190 in 1999 to approximately 600 in 2007. The rate of patients in DC treatment in Kosovo is 286 per million, lower than in other Central and Eastern European countries [4]. At the time of our study, patients were treated in six different dialysis centres (DC), with standard twice or three times a week five hour dialysis sessions (10% and 90%, respectively).We examined patients at the DC in Peja hospital which had no special areas dedicated to patients with positive history of hepatitis.

A number of reports have shown that viral hepatitis B (HBV) and viral hepatitis C (HCV) are common among ESRD patients [5-7]. In the dialysis centres of Kosovo and of other Eastern European countries, the prevalence of such infections has been poorly investigated. The few existing studies suggest that the prevalences are higher in patients dialysed in this part of Europe compared with other European countries [8-11]. The aim of this study was to

analyse the prevalence of viral hepatitis and other infections such as HIV, HVS-2, HHV-8 and syphilis in the ESRD patients of the hospital in the Peja region. Furthermore, we wanted to investigate whether the haemodialysis was associated with an elevated risk of infections. Our study was part of a survey carried out in the period 2004-2007 during a training project for healthcare workers at the hospital in the Peja region, supported by the Veneto Regional Health Authority and the Italian Co-operation Agency [12].

**Table 1**

**General characteristics of the 66 haemodialysis patients, compared to 452 non-haemodialysed patients (n=518)**

| Characteristics of patients | | Haemodialysis | | | | Chi-square homogeneity test |
|---|---|---|---|---|---|---|
| | | Yes | | No | | |
| | | N | % | N | % | p-value |
| Sex | Females | 27 | 41 | 296 | 65 | <0.01 |
| | Males | 39 | 59 | 156 | 35 | |
| Age | 18-30 | 3 | 4 | 185 | 41 | <0.01 |
| | 30-50 | 21 | 32 | 220 | 49 | |
| | 50+ | 42 | 64 | 47 | 10 | |
| Domicile | Urban | 21 | 32 | 173 | 38 | 0.31 |
| | Rural | 45 | 68 | 279 | 62 | |
| Education | ≤8 | 52 | 79 | 105 | 23 | <0.01 |
| | >8 | 14 | 21 | 347 | 77 | |
| Married | No | 8 | 12 | 165 | 63 | <0.01 |
| | Yes | 58 | 88 | 287 | 37 | |
| Employed | No | 42 | 64 | 109 | 24 | <0.01 |
| | Yes | 24 | 36 | 343 | 76 | |
| Blood transfusion | No | 2 | 3 | 442 | 98 | <0.01 |
| | Yes | 64 | 97 | 10 | 2 | |
| Dialysis (months) | 0-24 | 28 | 42 | - | - | - |
| | 24-48 | 13 | 20 | - | - | - |
| | 48+ | 25 | 38 | - | - | - |
| Pts. always in the same unit | No | 5 | 7 | - | - | - |
| | Yes | 61 | 93 | - | - | - |
| **Total** | | **66** | **100** | **452** | **100** | |

**Table 2**

**Seroprevalence of viral hepatitis, HIV, HSV-2, *Treponema pallidum* and HHV-8 of patients in haemodialysis, compared to non-haemodialysed patients**

| Serology | Haemodialysis | | | | Fisher's exact test p-value | Crude OR (95%CI) | Sex- and age-adjusted OR (95%CI) |
|---|---|---|---|---|---|---|---|
| | yes | | no | | | | |
| | N | % | N | % | | | |
| HAV[1] | 66 | 100 | 424 | 94 | 0.037 | NE | NE |
| HBsAg[2] | 14 | 21 | 16 | 3 | **<0.0001** | **7.34 (3.39,15.89)** | **5.18 (1.87,14.32)** |
| HBsAb[3] | 5 | 8 | 69 | 15 | 0.13 | 0.45 (0.18,1.17) | **0.27 (0.09,0.79)** |
| HBcAb[4] | 50 | 76 | 107 | 24 | **<0.0001** | **10.08 (5.51,18.42)** | **6.43 (3.22,12.85)** |
| HBV vax[5] | 2 | 3 | 0 | 0 | **0.016** | NE | NE |
| HDV[6] | 1 | 1 | 0 | 0 | 0.127 | NE | NE |
| HCV[7] | 57 | 86 | 3 | 1 | **<0.0001** | 947.89 (249.39,3602.83) | NE |
| HIV[8] | 0 | 0 | 0 | 0 | 1 | NE | NE |
| HSV-2[9] | 19 | 29 | 45 | 10 | **<0.0001** | **3.66 (1.98,6.77)** | **3.2 (1.46,7)** |
| *T. pallidum*[10] | 2 | 3 | 1 | 0.2 | **0.044** | **14.09 (1.26,157.66)** | NE |
| HHV-8[11] | 12 | 18 | - | - | - | - | - |

In bold: results significant at an alpha ≤ 0.05. Abbreviations used: OR: odds ratio; NE: not estimable; 1: hepatitis A virus; 2: hepatitis B surface antigen; 3: hepatitis B surface antibody; 4: hepatitis B core antigen: 5: HBV vaccinated subjects; 6: hepatitis delta virus; 7: hepatitis C virus; 8: human immunodeficiency virus: 9: human herpes virus 2; 10: Treponema pallidum; 11: human herpes virus 8

## Methods

Field work for this cross-sectional study was conducted from 1 January 2005 to 30 March 2005. The association between the prevalence of viral hepatitis and other infections and the haemodialysis status was assessed by comparing the ESRD patients with a group of blood donors and subjects who had been examined for routine laboratory testing. In addition, the scientific literature was reviewed to compare the HBV and HCV prevalence of patients in DC of different Eastern and Western European countries.

### Study population

All 66 ESRD patients treated at the DC of Peja regional hospital were enrolled in the study. Candidate blood donors being screened for donation suitability and individuals (18 years of age and older) who had undergone routine check-ups in two clinics in Peja and whose serum was sent for routine testing, were included in the study as a comparison group. In order to approximately randomise the group, patients screened on Monday, Wednesday and Friday were selected. In the three months of the study period, 285 blood donors and 187 subjects examined in clinics were potentially eligible for comparison. Out of the total number of 472 subjects, 20 refused to be tested or to respond to the questionnaire. The final number of 452 subjects was recruited. Approval from the Kosovo Institute of Public Health, the Regional Health Authorities and the Ethical Committee of the Peja region was obtained and a signed informed consent form from each participant was requested before entering the study.

**Table 3**

**HBsAg prevalence in haemodialysis centres in Western and Eastern European countries. Data on the general population is reported for comparison**

| Country | General population | Year | Reference | Haemodialysis Centres | Year | Reference |
|---|---|---|---|---|---|---|
| North European countries | | | | | | |
| Germany | 0.60% | 1998 | Thierfelder | 4.60% | 2001 | Burdick |
| UK | < 0.5% | 2001 | Eurohep | <0.5% | 2001 | Burdick |
| South European countries | | | | | | |
| Italy | 1% | 2001 | Eurohep | 4.30% | 2001 | Burdick |
| Spain | 1.70% | 2001 | Solà | 3.10% | 2001 | Burdick |
| Eastern European countries | | | | | | |
| Moldovia | 9% | 2004 | Emiroglu | 17% | 1999 | Covic |
| Romania | 6% | 2001 | Eurohep | 22% | 1998 | Vladutiu |
| Bulgaria | 5% | 2001 | Eurohep | - | - | - |
| Serbia | - | - | - | 15% | 1999 | Djukanovic |

**Table 4**

**HCV prevalence in haemodialysis centres in Western and Eastern European countries 1997-2001. Data on the general population is reported for comparison**

| Country | General population | Year | Reference | Haemodialysis centres | Year | Reference |
|---|---|---|---|---|---|---|
| North European countries | | | | | | |
| Germany | 0.60% | 1999 | Esteban | 3.80% | 2003 | Fissell |
| UK | 1% | 2001 | Bird | 2.60% | 2003 | Fissell |
| South European countries | | | | | | |
| Italy | 3.50% | 1997 | Esteban | 20.50% | 2003 | Fissell |
| Spain | 2.50% | 2001 | Dominguez | 22.90% | 2003 | Fissell |
| Eastern European countries | | | | | | |
| Moldavia | 5% | 1997 | Covic | 75% | 1999 | Covic |
| Romania | 6% | 2001 | Esteban | 73% | 1998 | Vladutiu |
| Bulgaria | 3% | 2001 | Esteban | 48% | 2008 | Atanasova |
| Poland | 2% | 2001 | Esteban | 44% | 1999 | Jadoul |
| Hungary | 0.50% | 2001 | Müller | 15% | 1999 | Jadoul |
| Serbia | - | - | - | 23% | 1999 | Djukanovic |

### Questionnaire

For all study participants information on socio-demographic characteristics and information related to haemodialysis treatment were collected by local physicians and nurses, interviewing patients using a structured questionnaire. The questionnaire included queries on age, sex, occupation, education, area of residence, partner status, length of dialysis treatment, number of transfusion received and if the patient remained always in the same unit of treatment. The serum was collected for laboratory investigations.

### Laboratory investigations

The collected serum was tested for the following hepatitis markers: total anti-HAV (IgG and IgM), HBsAg, anti-HBs, total anti-HBc (IgG and IgM), and anti-HCV using AxSYM microparticle enzyme immunoassay (MEIA) (Abbott Diagnostics, North Chicago IL). HBsAg-positive subjects were tested for antibodies to hepatitis delta virus (anti-HDV IgG) using a commercial enzyme-linked immunosorbent assay test (ELISA) (DiaSorin, Saluggia, Italy). A line immunoassay (LIA) (INNO-LIA HIV I/II Score, Innogenetics N.V., Gent, Belgium) was used for detecting antibodies to HIV type 1 and 2, and samples that were reactive were confirmed with Western blot. To detect anti-HSV-2 antibodies, a commercial HSV-2 specific IgG enzyme immunoassay (EIA) (HSV 2 IgG EIA WELL, Radim, Pomezia, Italy) was used. IgG and IgM antibodies to *Treponema pallidum* were detected by a *Treponema pallidum* recombinant EIA (Syphilis Screening Recombinant EIA WELL, Radim, Pomezia, Italy). HHV-8 serum antibodies were detected by a commercially available ELISA assay (HHV-8 IgG Elisa, Advanced Biotechnologies Incorporated, Columbia, MD, Unites States). All tests were performed according to the manufacturer's instructions at the Istituto Superiore di Sanità Laboratory, Rome, Italy, and partner institutions.

### Statistical analysis

Prevalence of viral hepatitis and other infectious diseases in haemodialysis patients was estimated and 95% confidence intervals (CI) calculated. We tested whether viral hepatitis and other infectious diseases were associated with haemodialysis by comparing seroprevalence in dialysis patients to seroprevalence in two comparison groups: blood donors and subjects who had been examined in clinics. At a first stage, association was tested separately in dialysis patients vs. blood donors, and in dialysis patients vs. patients who had been examined in clinics. Provided that the estimates were homogeneous in the two analyses, the two groups were pooled together to form a unique comparison group. To account for data sparseness, association was tested by means of Fisher's exact test. Odds ratios (OR) and 95% CI were calculated using logistic regression models. All statistical analyses were performed using R 2.8.0 [13].

## Results

Sixty-six haemodialysis patients were recruited (males: 59%, mean age: 55±14 years). The patient characteristics are reported in Table 1. The duration of haemodialysis treatment ranged from 12 to 264 months (median time 48 months). Concerning the aetiology of ESRD, glomerulonephritis was the first cause (20 cases, 30%), followed by diabetes mellitus (12 cases, 18%), pyelonephritis (9 cases, 14%), hypertension (7 cases, 10%), polycystic kidney diseases (4 cases, 6%), and systemic diseases (2 case, 3%). Aetiology was unknown for 12 cases (19%) of haemodialysis patients.

When comparing the distribution of hepatitis status in ESRD patients with subjects not undergoing haemodialysis, we found consistent results. Here we present results to the comparison between haemodialysis patients and the pooled group of comparison subjects. In total, 452 individuals (males: 35%, mean age: 34±11 years) were recruited for comparison. Participants' characteristics were all heterogeneous between haemodialysis and non-haemodialysis patients, except for the domicile (p-value=0.31) (Table 1).

### Serological status of dialysed patients

All ESRD patients were HAV positive indicating previous infection (Table 2). Prevalence of HBsAg, HBsAb, and HBcAb was 14 of 66, 21% (95%CI: 12-33%), 5 of 66, 8% (95%CI: 5-22%), and 50 of 66, 76% (95%CI: 64-85%), respectively. Two patients had been vaccinated for HBV. One male patient in his late forties was the only patient positive for HDV: he was also positive for HAV, HBV (HBcAb) and anti-HCV. HCV prevalence was 57 of 66, 86% (95%CI: 76-94%). Concerning the co-occurrence of HBV and HCV in haemodialysis patients, we observed that 45 (70%, 95%CI: 58-81%) were both HBV (HBcAb) and HCV, 10 (16%, 95%CI: 8-27%) had HCV but no HBV, five (8%, 95%CI: 3-17%) had HBV but no HCV, and four (6%, 95%CI: 2-15%) had none (Fisher's exact test p-value=0.096).

No HIV positive case was found. Prevalence of HSV-2 was 19 of 66, 29% (95%CI: 18-41%). Two patients (3%, 95%CI: 0-10%) were positive for *Treponema pallidum* and 12, 18% (95%CI: 10-30%) were HHV-8 positive.

HAV was associated with the haemodialysis status (Fisher's exact test p-value=0.037). Given that all dialysed patients were HAV positive, the estimation of OR was not possible. ESRD patients were at a higher risk of being HBsAg positive than others: sex- and age-adjusted OR was 5.18 (95%CI: 1.87-14.32). When additionally adjusting for the level of education, employment, marital status, and domicile, the OR increased up to 7.92 (95%CI: 2.31-27-12). HBcAb positivity was strongly associated with haemodialysis: the sex- and age-adjusted OR was 6.43 (95%CI: 3.22-12-85); it increased slightly when further adjusting for education, employment, marital status, and domicile as well to OR 6.9 (95%CI: 3.17-15.03). HCV prevalence was 86% and 1% in presence and absence of haemodialysis treatment, respectively. For ESRD patients and the comparison group an OR could not be calculated. However, the Fisher's exact test for association proved a strong association between haemodialysis and HCV (p-value<0.0001). The OR for association between haemodialysis and HSV-2 positivity was 3.20 (95%CI: 1.46-7.00) when adjusting for sex and age, and rising up to 6.44 (95%CI: 2.40-17.27) when further adjusting for education, employment, marital status, and domicile. Significant associations were also observed between haemodialysis status and *Treponema pallidum* status (Fisher's exact test p-value=0.044). Results of the association study are reported in Table 2.

### Prevalence of HBV and HCV in DC of Eastern and Western European countries

Table 3 shows the prevalence of serological markers for HBV in DC of Northwestern European countries, Southwestern European countries and Eastern European countries [8,10,14,15]. Table 4 shows the difference for HCV prevalence among DC in Northwestern European countries (UK, Germany) Southwestern European countries (Italy, Spain) and different Eastern European countries [8-11,14,16].

In the majority of the Eastern countries, the prevalence is over 40%, with more than 70% in Romania and Moldavia. Unlike HBV the HCV prevalence in the general population of Eastern countries is in some cases lower than in Western countries (Table 4) [8, 22-25].

## Discussion

The prevalence of viral hepatitis and other agents among ESRD patients in the current study indicates a very high level of endemicity. Twenty-one per cent of patients were found to be HBsAg carriers and more than 78% had been exposed to the virus (anti-HBc positive), with a sex- and age-adjusted six-fold risk when compared to non-haemodialysis patients. In analysing the data in the literature, it was found that approximately 20% of dialysis patients are chronic carriers of HBV in Eastern Europe, compared to approximately 4 % in Western countries. On the other hand, the general population carriage is at least three times higher than in Western Europe (Table 3) [17-20].

Kosovo is a country with a low prevalence of HCV infection [12,21]. This was reflected in the group of non-hemodialysis patients, where the prevalence of HCV was as low as 1%. Nevertheless, the prevalence of HCV in dialysed patients was strikingly high (86%). It was not possible to calculate the OR with the observed numbers. However, the great difference should suggest that, even taking into account potential differences between the two groups compared in this study, hemodialysis should be considered a strong risk factor for HCV infection, in line with the results of other studies carried out in Eastern European countries [8-10,15]. The HCV prevalence in DC in Western versus Eastern countries differs: from around 2% in Germany and the United Kingdom (UK) to 20% in Spain and Italy and up to 50-70% in Eastern European countries [8,15]. In the Dialysis Outcomes and Practice Patterns Study (DOPPS), the mean prevalence of HCV infection in five Western European countries (France, Germany, Italy, Spain and the UK), Japan and the United States (US) was 13% [16].

In Europe, the overall prevalence of HBV and HCV in ESRD patients has been decreasing over the years as a result of HBV vaccination, routine screening of blood products, and the use of recombinant human erythropoietin [6,11,16]. Guidelines for universal precautions - 'Recommendations for preventing transmission of infections among chronic hemodialysis patients' - had been initially recommended by the US Centers for Disease Control and Prevention (US, CDC) in 1985 and successively updated [26]. In Kosovo, erythropoietin started to be used in 2004 but with marked differences between centres. The percentage of haemodialysis patients receiving erythropoietin in Kosovo is, to date, less than 50%. The situation appears to be improving slightly, but precise figures are not available. Screening of blood-donors for blood-borne viruses has only been implemented regularly since 2001. No immunisation policy for hepatitis vaccination existed in general in Kosovo before the war. In Kosovo there is the policy for HBV vaccination of haemodialysis patients and medical staff. The lack of available vaccines hampers its implementation; for example, in 2005 the percentage of vaccinated individuals among the 253 health care workers of the Peja hospital was 16.6% [12]. An important measure for the control of hepatitis infection is the segregation of positive patients and their haemodialysis equipment [27]. Until recently, the lack of resources prevented this practice in Kosovo.

In our study the syphilis prevalence (anti-*Treponema pallidum* IgG) among dialysed patients was 3%, much higher than the 0.2% of non-dialysed subjects. There is little data on syphilis seroprevalence in DC patients. Sexual contact is the primary mode of transmission of syphilis, but blood transfusion, blood contact and accidental inoculations are other modes of infection that place ESRD patients at risk. A report from Taiwan showed a prevalence of syphilis among dialysed patients of 5.6% [28]. In a more recent study, the syphilis seroprevalence in 167 ESRD patients was 6.7%, more than two times higher than the overall prevalence reported in the general population [29].

HHV-8 is a gamma-herpes virus, closely related to the Epstein-Barr virus. We do not have data to compare our study population with the Kosovar general population. Nevertheless, in nearby Albania, HHV-8 seroprevalence in the general population is reported to be 20% [30]. Transmission of HHV-8 infection through blood, although suggested, is controversial. A case-control study performed in 97 dialysed patients from Northern Italy found a prevalence of 9.2% (in this geographic area the prevalence of HHV-8 in blood donors was 12.7%) [31]. In Greece, HHV-8 prevalence in 485 dialysed patients was 7.2% [32]. In Southern Italy, the seroprevalence of HHV-8 among ESRD patients was 27% (comparable to 25% as observed in the general population) [33].

In Kosovo, the prevalence of infection from viral hepatitis,HHV-8, HSV-2 and Treponema pallidum among ESRD patients is high, indicating major ongoing nosocomial transmission. Even though this may be a consequence of limited resources available, targeted recommendations could be implemented to improve the current situation:

- rigorous attention should be paid to infection control procedures such as changing gloves between patients and the decontamination of equipment and surfaces after each patient treatment episode;
- all single-use injectable medications and solutions should used on a single patient, and all parenteral medications should be prepared in a clean area separate from potentially contaminated items and surfaces;
- hepatitis B vaccination should be given to all patients and staff [34];
- HBsAg and HCV positive patients and their dialysis equipment should be segregated;
- periodic diagnostic testing of patients and healthcare workers needs to be carried out;
- dialysis providers should be aware of their responsibility to report clusters of infections to the local health authorities, as the failure to report illness clusters can result in delays in the recognition of disease outbreaks; and
- training for health care workers should be implemented periodically.

Our study has several limitations that have to be emphasised. As the data were restricted to one DC, the results presented here cannot be considered indicative for Kosovo as a whole and figures on serological status of the health personnel are not available. Furthermore data on the incidence of infectious diseases after the regular screening of blood transfusion for blood-borne viruses were implemented (2001) are not available; and information on possible risk factors is also missing. In Kosovo further studies on the prevalence and incidence of blood borne viruses among ESRD patients are needed, involving more than one DC, and exploring possible risk factors in these patients and settings.

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