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Original Article

Molecular diagnosis of infectious diseases in São Miguel Island (Azores, Portugal): A hospital-based descriptive study

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Abstract

Introduction: We performed a descriptive analysis of molecular diagnosis of infectious agents in the São Miguel Island population, in order to address questions like what is the frequency of clinical requests, is it observable seasonality of pathogens, and what is the positive rate for the clinical diagnosis.

Methodology: This was a retrospective and descriptive study based on 878 individuals suspected of harboring infectious diseases during two consecutive years, 2012–2013. More than 25 different pathogens were investigated by polymerase chain reaction (PCR)-based methods. The individuals were stratified into gender, occupation, and age groups.

Results: The pathogen with more clinical requests was hepatitis C virus, investigated in 225 individuals (30.0%), followed by *Leptospira* spp., in 187 (24.9%). Overall, data demonstrated a gender distribution bias, where 72.9% of cases were males. The age group of 25 to 44 years was the class with more clinical requests. Regarding occupation, a predominance of construction workers (12.0%) was observed, followed by retired workers (11.0%). Patient distribution per year showed a higher number of patients in the fall months. Diagnoses of leptospirosis and respiratory virus infections presented seasonality.

Conclusions: The present study provides a valid contribution to the knowledge of the epidemiology of infectious diseases in the São Miguel Island (Azores, Portugal) population.

Key words: communicable diseases; leptospirosis; herpesvirus; hepatitis; respiratory viruses; Azores.

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Introduction

The Hospital of Divino Espirito Santo of Ponta Delgada (HDES) is the main hospital in the Azores archipelago of Portugal. After transferring to a new building in 2000, it received the full accreditation by the UK-CHKS in 2007. Currently, it has 35 clinical specialties, being for some the reference center of the Azores. The HDES is also a teaching hospital for undergraduate medical students from the University of Azores. The hospital serves around 144,000 individuals (58% Azorean population). With the mission to provide differentiated healthcare, HDES is committed to setting the standard for excellence in patient care and safety, providing exemplary clinical settings for educating healthcare professionals, and introducing innovative methodologies for healthcare delivery and quality improvement. With these objectives in mind, in 2001, the Molecular Genetics and Pathology Unit (UGPM) was created. This unit is responsible for implementing molecular tests for diagnosis, prognosis and therapeutic monitoring, as well as performing clinical research relevant to the needs of the Azorean regional health system. The UGPM primarily performed molecular diagnosis and investigation of human genetic diseases. Afterwards, considering the UGPM's knowledge on molecular techniques and the fact that conventional microbiology methods for identifying pathogens are time-consuming and often have a limited impact on early therapeutic decisions, the lab implemented molecular methods for the detection of infectious agents in the clinical context.

The first molecular test for infectious disease to be implemented, in 2005, was the detection of *Leptospira* spp., a fastidious bacterium that causes leptspirosis, a

severe disease leading to systemic infection. The microscopic agglutination test (MAT) is the gold standard for serodiagnosis of leptospirosis, but detectable titers of antibodies appear in the blood about 5–10 days after the onset of disease [1]. Consequently, the polymerase chain reaction (PCR)-based test allowed the reduction in the time of bacteria detection from 15 days to about 6 hours (by conventional nested PCR), which contributes greatly to providing better healthcare. Since then, the lab increased its offer on molecular tests; for example, in the period 2012–2013, the UGPM performed a total of 3,922 analyses, the majority being the detection of infectious agents (3,141; 80.1%). The remaining molecular diagnoses (781; 19.9%) were genetic disorders, with HLA (human leukocyte antigen) genotyping being the most commonly requested (34.1%).

Infectious diseases are closely dependent on the nature and complexity of human behavior, as they reflect who we are, what we do, and how we interact with other people, animals, and the environment. More recent changes in the global climate and environment have had a dramatic effect on the frequency of certain infectious diseases [2]. Considering that the Azores is an archipelago with a semi-tropical climate and that HDES is a medium-sized island hospital (381 beds), we present a descriptive analysis of the molecular diagnosis of infectious agents in the São Miguel Island population, in order to address questions such as what is the frequency of clinical requests, is a seasonality of pathogens observable, and what is the positive rate for the clinical diagnosis.

Methodology

Ethics statement

The present investigation (ref. HDES/CES/2013/371) was approved by the health ethics committee of the Hospital of Divino Espírito Santo of Ponta Delgada, EPER. Data collection followed the ethical principles for human research, which include human rights and privilege, preservation of dignity and integrity of man, as well as privacy and confidentiality of data.

São Miguel Island population (Azores) bio-demographic data

São Miguel is the largest (747 km^2, measuring 64 km from east to west, and 8 to 15 km wide) and the most populated Azorean island (137,699 inhabitants; 55.9% total Azores population; Portugal Census, 2011). This island was uninhabited when it was originally discovered in 1427. Around half of the São Miguel population lives in small rural localities, which are characterized by agriculture and a cattle-breeding economy, and its inhabitants show great similarity in lifestyle, as well as in eating habits.

Study design

The current work is a retrospective and descriptive study based on 878 individuals harboring or suspected of harboring infectious diseases during two consecutive years, 2012–2013 (Table 1). These individuals were treated in the departments of clinical inpatient and outpatient (emergency room and outpatient consultations) at the HDES. The detection of the pathogens was performed by PCR-based methods, which are described in Supplementary Table 1. The results were expressed as follows: positive (when the pathogen was detected), negative (when the pathogen was not detected), and inconclusive (unable to resolve due to, for example, sample inhibition, pathogen load, or to unknown reason).

Study groups

Molecular techniques aided in confirming the presence of a pathogen in people with clinical suspicion of causing infection. At the UGPM, clinical samples of 878 individuals in the situations indicated in Table 1 were analyzed to confirm the presence of a specific pathogen (diagnosis group; 729 individuals), to monitor the presence of a particular pathogen or its drug sensitivity and resistance (monitoring group; 21 individuals), and to perform differential diagnoses of infectious diseases (differential diagnosis group; 128 individuals). Considering that, for some pathogens, a reduced number of molecular tests was performed, only the most commonly requested ($n \geq 10$) tests are presented and discussed, *i.e.*, *Leptospira* spp., *Mycobacterium tuberculosis* and rifampicin resistance, hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus (CMV), herpes simplex virus (HSV1 and HSV2), influenza virus [A, A(H1N1) pdm09, and B], and sepsis (Table 1). These inclusion criteria reduced the diagnosis group sample to 683 (82.1%) and, consequently, the study sample to 832 individuals (Table 2).

The variables evaluated were age, gender, occupation, type of molecular diagnosis requested, specimen used, date of reception of the samples by the laboratory, and the date of communication of the clinical results. Results for some of these variables were not presented because, in some cases, there was no relevant information for the purpose of the present

work. The individuals were stratified into gender (male/female), occupation (farmers, housewives, construction workers, retired, carpenters, students, unemployed, public employees, nurses, physicians, among others), and six standardized age groups, namely young children (0–4 years of age), children and adolescents (5–14 years), young adults (15–24 years), adults (25–44 years), older adults (45–64 years old), and elderly (≥ 65 years of age).

Statistical analysis

Descriptive statistics, which included frequencies and means, were calculated using SPSS software version 20 (SPSS, Chicago, USA) for Windows. The normal distribution of variables was examined using the Kolmogorov-Smirnov test. Chi-square tests (χ^2) were calculated in order to assess independence of the evaluated variables. The statistical significance level adopted for all estimates was $p < 0.05$.

Mean annual incidence was calculated for leptospirosis and tuberculosis by the following formula: average number of new cases during the time period/population at risk during the same time period (São Miguel Island population).

Results

The UGPM, located at the main Azorean hospital, does not perform conventional microbiology techniques; but, due to installed know-how, molecular methods for the detection of infectious agents in the clinical context have been implemented. Samples from 878 individuals suspected of having an infectious disease were analyzed over a two-year period. More than 25 different pathogens were investigated (Table 1). The pathogen with more clinical requests was HCV, evaluated in 225 individuals (30.0%), followed by *Leptospira* spp., in 187 (24.9%). Of a total of 704 individuals, corresponding to diagnosis and monitoring

Table 1. Overview of individuals harboring or suspected of harboring infectious diseases based on diagnosis and monitoring groups.

Molecular tests for infectious diseases		**Diagnosis**		**Monitoring**		**Total**	
Pathogens	**ID**	**N**	**(%)**	**N**	**(%)**	**N**	**(%)**
Bacteriology	**A**						
***Leptospira* spp.**	A1	**187**	**(24.9)**	–		**187**	**(24.9)**
***Mycobacterium tuberculosis* and rifampicin resistance**	A2	**97**	**(12.9)**	–		**97**	**(12.9)**
Mycoplasma pneumoniae	A3	7	(0.9)	–		7	(0.9)
Bordetella pertussis/parapertussis	A4	5	(0.7)	–		5	(0.7)
Chlamydia trachomatis	A5	4	(0.5)	–		4	(0.5)
Brucella spp.	A6	2	(0.3)	–		2	(0.3)
Neisseria gonorrhoeae	A7	1	(0.1)	–		1	(0.1)
*MRSA (*methicillin-resistant *Staphylococcus aureus)*	A8	1	(0.1)	–		1	(0.1)
Coxiella burnetii	A9	1	(0.1)	–		1	(0.1)
Legionella pneumophila	A10	1	(0.1)	–		1	(0.1)
Virology	B						
Hepatitis virus							
– **Hepatitis C virus (HCV)**	**B1**	**214**	**(28.5)**	**11**	**(1.5)**	**225**	**(30.0)**
– **Hepatitis B virus (HBV)**	**B2**	**25**	**(3.3)**	**1**	**(0.1)**	**26**	**(3.5)**
Herpesvirus							
– **Cytomegalovirus (CMV)**	**B3**	**78**	**(10.4)**	**8**	**(1.1)**	**86**	**(11.5)**
– **Herpes simplex virus (HSV-1 and HSV-2)**	**B4**	**14**	**(1.9)**	**1**	**(0.1)**	**15**	**(2.0)**
– Herpesvirus family (8 virus)	B5	8	(1.1)	–		8	(1.1)
– Epstein-Barr virus (EBV)	B6	5	(0.7)	–		5	(0.7)
– Varicella Zoster (VZV)	B7	2	(0.3)	–		2	(0.3)
– Human herpesvirus 8 (HHV-8)	B8	1	(0.1)	–		1	(0.1)
Respiratory virus							
– **Influenza virus [A, A(H1N1)pdm09, B]**	**B9**	**55**	**(7.3)**	–		**55**	**(7.3)**
– Enterovirus	B10	3	(0.4)	–		3	(0.4)
– Adenovirus	B11	1	(0.1)	–		1	(0.1)
Others							
– Parvovirus B19	B12	3	(0.4)	–		3	(0.4)
– Polyomavirus (JC and BK)	B13	1	(0.1)	–		1	(0.1)
Other	C						
Sepsis	**C1**	**13**	**(1.7)**	**–**		**13**	**(1.7)**
Total		729	(83.0)	21	(2.4)	750	(85.4)

Bold represents the molecular tests most commonly requested (n > 10).

groups, 402 (57.1%) presented a negative result; in 292 (41.5%), a pathogen was identified, and in 10 (1.4%) individuals the result was inconclusive. Overall, the data demonstrated gender distribution bias, where 72.9% (615 out of 832 individuals) were males and 25.7% (217 out of 832) were females. Considering age distribution, the data showed that people between 25 and 44 years of age, followed by older adults (45–64 years), were the classes with more clinical requests (Table 2). Regarding occupation, a predominance of

Table 2. Distribution of individuals tested according to age, gender, and occupation, considering only the molecular tests most commonly requested (n ≥ 10).

Demographic characteristics	Diagnosis group		Monitoring group		Diff. diagnosis group		Total	
	N	(%)	N	(%)	N	(%)	N	(%)
Age classes (years)								
– Young children (0–4)	36	(4.3)	2	(0.2)	16	(1.9)	54	(6.4)
– Children and adolescents (5–14)	7	(0.8)	0	(0.0)	8	(0.9)	15	(1.8)
– Young adults (15–24)	47	(5.6)	1	(0.1)	10	(1.2)	58	(6.9)
– Adults (25–44)	295	(35.4)	12	(1.4)	35	(4.2)	342	(40.5)
– Older adults (45–64)	193	(23.2)	4	(0.5)	38	(4.6)	235	(27.8)
– Elderly (≥ 65)	105	(12.6)	2	(0.2)	21	(2.5)	128	(15.2)
Gender								
– Female	173	(20.8)	3	(0.4)	41	(4.9)	217	(25.7)
– Male	510	(61.3)	18	(2.2)	87	(10.4)	615	(72.9)
Occupation								
– Farmers	46	(5.5)	2	(0.2)	4	(0.5)	52	(6.2)
– Housewives	57	(6.8)	2	(0.2)	12	(1.4)	71	(8.4)
– Construction workers	90	(10.8)	2	(0.2)	9	(1.1)	101	(12.0)
– Retired	82	(9.8)	0	(0.0)	11	(1.3)	93	(11.0)
– Carpenters	15	(1.8)	0	(0.0)	1	(0.1)	16	(1.9)
– Students	61	(7.3)	1	(0.1)	19	(2.3)	81	(9.6)
– Unemployed	34	(4.1)	3	(0.4)	8	(0.9)	45	(5.3)
– Public employees	20	(2.4)	2	(0.2)	3	(0.4)	25	(3.0)
– Nurses	5	(0.6)	0	(0.0)	3	(0.4)	8	(0.9)
– Physicians	3	(0.4)	0	(0.0)	1	(0.1)	4	(0.5)
– Others	270	(32.4)	9	(1.1)	57	(6.8)	336	(39.8)
Total	**683**	**(82.1)**	**21**	**(2.5)**	**128**	**(15.4)**	**832**	**(100.0)**

Table 3. Characterization of individuals tested for *Leptospira* spp. and *Mycobacterium tuberculosis*.

Demographic characteristics	*Leptospira* spp. (N=187)				*Mycobacterium tuberculosis* (N=97)			
	Positive (N=88)		Negative (N=99)		Positive (N=12)		Negative (N=85)	
	N	(%)	N	(%)	N	(%)	N	(%)
Age (years)								
– Young children (0–4)	0	(0.0)	0	(0.0)	0	(0.0)	3	(3.1)
– Children and adolescents (5–14)	0	(0.0)	0	(0.0)	0	(0.0)	2	(2.1)
– Young adults (15–24)	6	(3.2)	7	(3.7)	0	(0.0)	3	(3.1)
– Adults (25–44)	38	(20.3)	30	(16.0)	5	(5.1)	24	(24.7)
– Older adults (45–64)	30	(16.0)	37	(19.8)	6	(6.2)	32	(33.0)
– Elderly (≥ 65)	14	(7.5)	25	(13.4)	1	(1.0)	21	(21.6)
Gender								
– Female	11	(5.9)	20	(10.7)	4	(4.1)	31	(31.9)
– Male	77	(41.2)	79	(42.2)	8	(8.2)	54	(55.7)
Occupation								
– Farmers	21	(11.2)	15	(8.0)	0	(0.0)	0	(0.0)
– Physicians	0	(0.0)	0	(0.0)	0	(0.0)	3	(3.1)
– Construction workers	9	(4.8)	6	(3.2)	3	(3.1)	8	(8.2)
– Retired	10	(5.3)	21	(11.2)	2	(2.1)	15	(15.5)
– Others	48	(25.7)	57	(30.5)	7	(7.2)	59	(60.8)

construction workers (12.0%) was observed, followed by retired workers (11.0%) and students (9.6%; Table 2). Patient distribution per year showed a higher number of patients in the fall months (October to December).

Molecular diagnosis of leptospirosis

Leptospirosis is an endemic disease considered a public health problem in the Azores Islands (Portugal). Here, 187 patients were tested for this disease, and 88 (47.1%) were infected with *Leptospira* spp. (Table 3). The mean age for these patients was 45.7 ± 16.3 years. In order to observe if the detection of *Leptospira* spp. was independent of variables such as age, gender, and occupation, a Chi-square test was applied. The data showed that case distribution was influenced by these variables ($p < 0.0001$). For instance, the mean age of female individuals was significantly higher than the mean male age (55.8 ± 18.8 years versus 44.3 ± 15.6 years). Simultaneously, males (41.2%) and farmers (11.2%) were the most affected by this pathogen (Table 3).

The analysis of positive sample distribution over time demonstrated a higher number of leptospirosis cases in the third trimester of 2012 and in the second trimester of 2013. Regarding mean annual incidence, a value of 32.9/100,000 inhabitants in the 2012–2013 period is estimated. Considering the type of sample collected, the data demonstrated a higher number of positive results in urine (34; 18.2%) compared with serum (20, 10.7%).

Molecular diagnosis of tuberculosis

Tuberculosis (TB) continues to be one of the most devastating and widespread infections in the world. The data showed that in 12 (12.4%) of 97 patients, the *Mycobacterium tuberculosis* complex (MTB) was detected: 10 in respiratory samples (out of 49) and 2 in lymphatic nodes (out of 7). Overall, the mean annual incidence of TB was 4.7/100,000 (3.9/100,000 for pulmonary TB and 0.7/100,000 for extrapulmonary TB). Considering gender distribution, the results showed a higher proportion of males (61.5%) affected by MTB, who had a mean age of 48.9 ± 11.1 years, a value higher than that of females (40.0 ± 28.1 years).

Considering that the molecular test implemented in the lab (Xpert MTB/RIF, Solna, Sweden, Table 1-S) only determines resistance to rifampicin (RIF), the results showed that, out of 12 individuals who tested positive, 8 presented MTB sensitive to rifampicin, and 4 showed a RIF-resistant genotype. Of these, only one was sensitive to isoniazid; the remaining three were resistant to both isoniazid and RIF.

Molecular diagnosis of hepatitis B and C virus

Viral hepatitis has emerged as a major public health problem throughout the world, affecting hundreds of millions of people. A total of 251 individuals were tested for viral hepatitis caused by HBV and HCV. In 163 (64.9%) of them, the pathogens were identified; 149 (59.4%) had HCV, 12 (4.8%) HBV, and 2 showed HBV/HCV co-infections.

Considering HBV infections, of the 26 individuals evaluated, 12 (46.2%) tested positive. The majority of these individuals were adults between 25 and 44 years of age (Table 4), and were equally distributed by gender. HBV genotype results showed the presence of only three genotypes (A, D, and E), with genotype D being the most frequent, with seven cases (63.6%), followed by genotypes A and E, both with 18.3%. Males presented higher frequency of genotypes D and A, whereas females more frequently showed genotype E.

Regarding HCV infection, 149 individuals tested positive for this virus, with a mean age of 33.9 ± 9.7 (varying from 5 to 67 years) (Table 4). Gender distribution showed a male/female ratio of 8.3/1. HCV genotypes analysis demonstrated that genotype 1 was the most common (62.4%), followed by genotype 3 (18.1%). Nevertheless, genotype 2 (2.0%) was more prevalent than was genotype 4 (0.7%). Genotype 1 sub-typing results revealed a predominance of genotype 1a with a frequency of 61.1%. Only two individuals presented genotype 1b. During the data collection period, two individuals presented infection by two different HCV viruses in two different time points, and another five patients (3.4%) had a co-infection. In these last patients, the following combinations of different HCV virus were observed: 1a/1b, 1a/3, 4c/4d, 4/1b/1a. To determine the rate of mother-to-child transmission of HCV, 13 infants born to HCV-positive mothers were studied; only one (7.7%) infant presented the virus.

Molecular diagnosis of herpesvirus family

Herpesviruses are large double-stranded DNA viruses affecting not only mammals, but also frogs, lizards, birds, fish, and mosquitoes. In humans, they cause a broad spectrum of clinical manifestations. In the present study, a total of 199 individuals were tested for the presence of herpesvirus nucleic acids, with the majority (85%) being negative. The most requested test was CMV detection (n = 86), followed by the simplex viruses 1 and 2 (n = 15; Table 5). The remaining

individuals (n = 98) were tested for the detection of other infectious agents (differential diagnosis). In this last group, 16 individuals presented a herpesvirus: three HSV1/2, one varicella-zoster virus (VZV), four Epstein-Barr virus (EBV), one CMV, one human herpesvirus 6 (HHV6), four human herpesvirus 7 (HHV7), one co-infection of EBV and HHV6, and finally one coinfection of EBV, HHV6, and HHV7.

With respect to CMV infection, a total of 10 individuals were infected with this virus, the majority (n = 6) being elderly (Table 5), with a mean age of 76 ± 6.3 years. Congenital CMV was tested in 18 children, with one resulting positive (5.5%). Three patients were monitored to prevent a CMV infection after organ transplantation, and none presented any positive test results for the virus.

Concerning HSV1/2, the results showed that, of the fifteen individuals tested, the HSV1 virus was detected in only two (Table 5). In these two patients, the samples analyzed were skin lesions. The HSV1/2 viruses were also evaluated in combination with other infectious agents, for differential diagnosis; cerebrospinal fluid

Table 4. Characterization of individuals tested for HBV and HCV.

Demographic characteristics	HBV (N=26)						HCV (N=225)					
	Positive (N=12)		Negative (N=13)		Inconclusive (N=1)		Positive (N=149)		Negative (N=67)		Inconclusive (N=9)	
	N	(%)	N	(%)	N	(%)	N	(%)	N	(%)	N	(%)
Age												
– Young children (0–4)	0	(0.0)	0	(0.0)	0	(0.0)	3	(1.3)	10	(4.4)	1	(0.4)
– Children and adolescents (5–14)	0	(0.0)	0	(0.0)	0	(0.0)	1	(0.4)	0	(0.0)	0	(0.0)
– Young adults (15–24)	2	(7.7)	1	(3.8)	0	(0.0)	18	(8.0)	5	(2.2)	1	(0.4)
– Adults (25–44)	7	(26.9)	7	(26.9)	1	(3.8)	105	(46.7)	37	(16.4)	3	(1.3)
– Older adults (45–64)	2	(7.7)	5	(19.2)	0	(0.0)	21	(9.3)	14	(6.2)	4	(1.8)
– Elderly (≥ 65)	1	(3.8)	0	(0.0)	0	(0.0)	1	(0.4)	1	(0.4)	0	(0.0)
Gender												
– Female	6	(23.1)	0	(0.0)	0	(0.0)	16	(7.1)	22	(9.8)	1	(0.4)
– Male	6	(23.1)	13	(50.0)	1	(3.8)	133	(59.1)	45	(20.0)	8	(3.5)
Occupation												
– Construction workers	2	(7.7)	5	(19.2)	0	(0.0)	33	(14.7)	14	(6.2)	2	(0.9)
– Carpenters	0	(0.0)	1	(3.8)	0	(0.0)	3	(1.3)	2	(0.9)	0	(0.0)
– Students	1	(3.8)	1	(3.8)	1	(3.8)	20	(8.9)	1	(0.4)	1	(0.4)
– Unemployed	1	(3.8)	1	(3.8)	0	(0.0)	17	(7.5)	5	(2.2)	1	(0.4)
– Others	8	(30.8)	5	(19.2)	0	(0.0)	76	(33.8)	45	(20.0)	5	(2.2)

Table 5. Characterization of individuals tested for CMV and HSV1/HSV2.

Demographic characteristics	CMV (N=86)				HSV1/HSV2 (N=15)			
	Positive (N=10)		Negative (N=76)		Positive (N=2)		Negative (N=13)	
	N	(%)	N	(%)	N	(%)	N	(%)
Age								
– Young children (0–4)	1	(1.2)	17	(19.8)	0	(0.0)	3	(20.0)
– Children and adolescents (5–14)	0	(0.0)	0	(0.0)	0	(0.0)	2	(13.3)
– Young adults (15–24)	0	(0.0)	4	(4.6)	0	(0.0)	0	(0.0)
– Adults (25–44)	0	(0.0)	35	(40.7)	1	(6.7)	4	(26.6)
– Older adults (45–64)	3	(3.5)	19	(22.1)	0	(0.0)	1	(6.7)
– Elderly (≥ 65)	6	(6.9)	1	(1.2)	1	(6.7)	3	(20.0)
Gender								
– Female	3	(3.5)	29	(33.7)	1	(6.7)	7	(46.6)
– Male	7	(8.1)	47	(54.7)	1	(6.7)	6	(40.0)
Occupation								
– Public employees	1	(1.2)	2	(2.3)	1	(6.7)	0	(0.0)
– Housewives	1	(1.2)	7	(8.1)	0	(0.0)	1	(6.7)
– Students	0	(0.0)	7	(8.1)	1	(6.7)	4	(26.6)
– Retired	3	(3.5)	0	(0.0)	0	(0.0)	3	(20.0)
– Others	5	(5.8)	60	(69.8)	0	(0.0)	5	(33.3)

(45%) and serum (27.5%) were the most common biological samples tested. In these samples, only three individuals with HSV1 were identified, all of whom were > 63 years of age and immunologically compromised.

Molecular diagnosis of respiratory virus

Influenza, commonly known as "the flu", is an infectious disease caused by an influenza virus with symptoms that can be mild to severe. The influenza viruses A, A(H1N1) pdm09, and B were the most commonly requested, with 55 individuals analyzed (6.9%; Table 6). Of these, six were infected by influenza virus, three with influenza A virus, and three with influenza B virus, with a distribution in the winter and spring months. No individuals were found to be infected with the A(H1N1) pdm09 virus. All infected individuals were older, with average age of 53.0 ± 11.9 years.

In seven patients with symptoms of upper and lower respiratory tract infections, the physicians requested the search for a panel of respiratory viruses, which included, along with the above-described influenza viruses, the following: rhinovirus, coronavirus (NL63, 229E, OC43, HKU1), parainfluenza (1, 2, 3, 4), human metapneumovirus (A/B), bocavirus, respiratory

Table 6. Characterization of individuals tested for influenza virus A, A(H1N1)pdm09, and B.

Demographic characteristics	Positive (N=6)				Negative influenza virus (N=49)	
	Influenza A (N=3)		Influenza B (N=3)			
	N	(%)	N	(%)	N	(%)
Age						
– Young children (0–4)	0	(0.0)	0	(0.0)	0	(0.0)
– Children and adolescents (5–14)	0	(0.0)	0	(0.0)	0	(0.0)
– Young adults (15–24)	0	(0.0)	0	(0.0)	3	(5.4)
– Adults (25–44)	0	(0.0)	1	(1.8)	8	(14.5)
– Older adults (45–64)	1	(1.8)	2	(3.6)	15	(27.3)
– Elderly (≥ 65)	2	(3.6)	0	(0.0)	23	(41.9)
Gender						
– Female	0	(0.0)	1	(1.8)	18	(32.7)
– Male	3	(5.4)	2	(3.6)	31	(56.4)
Occupation						
– Architects	0	(0.0)	1	(1.8)	0	(0.0)
– Technicians	1	(1.8)	0	(0.0)	0	(0.0)
– Nurses	0	(0.0)	0	(0.0)	3	(5.5)
– Retired	0	(0.0)	0	(0.0)	19	(34.6)
– Others	2	(3.6)	2	(3.6)	27	(49.1)

Table 7. Polymerase chain reaction-positive sepsis profiles.

Pathogens	Patients' sepsis profiles										
	A (n=1)	B (n=2)	C (n=1)	D (n=1)	E (n=1)	F (n=1)	G (n=1)	H (n=2)	I (n=1)	J (n=1)	K (n=1)
Gram-positive bacteria											
Streptococcus spp.		+					+		+		
Enterococcus spp.			+	+	+		+	+	+		
Staphylococcus spp.	+		+	+					+		
Drug resistance											
vanA (vancomycin)	S	S	R	R	S		S	S	S		
vanB (vancomycin)	S	S	S	R	S		S	S	S		
mecA (methicillin)	R	S	R	S	S		S	S	R		
Gram-negative bacteria											
Gram-negative A[1]	+			+			+		+	+	+
Gram-negative B[2]	+		+	+		+		+	+	+	+
Fungi											
Fungi[3]	+										

[1] Gram-negative A includes: *Pseudomonas aeriginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophila*, *Serratia marcescens*, *Bacteroides fragilis*, and *Salmonella typhi*; [2] Gram-negative B includes: *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Escherichia coli*, *Enterobacter cloacae*, and *Enterobacter aerogenes*; [3] Fungi includes: *Candida albicans*, *Candida tropicalis*, *Candid parapsilosis*, *Candida glabrata*, *Candida krusei*, and *Aspergillus fumigatus*.

syncytial virus (A/B), adenovirus, enterovirus, and parechovirus (for molecular tests, see Table 1-S). The results showed that only two individuals were negative for these viruses. Three patients presented the following viruses: one respiratory syncytial virus (A/B), one rhinovirus, and one influenza B. On the other hand, two showed coinfections: one with adenovirus, bocavirus, and parainfluenza; and one with influenza B, adenovirus, rhinovirus, bocavirus, and parainfluenza.

Molecular diagnosis of sepsis

Severe sepsis and septic shock are major healthcare problems, affecting millions of people around the world each year. In total, 13 individuals were molecularly tested for sepsis, and in all of them, a pathogenic agent was detected, in a co-infection situation the majority of the time (Table 7). The mean age of these individuals was 63.9 ± 14.9 years, with the youngest patient age being 35 years of age. Males (n = 7) and females (n = 6) were equally affected; however, females were older than males (67.2 ± 14.4 years versus 61.1 ± 17.2 years, respectively). Moreover, the majority of patients (9 of 13) were inpatients of the ICU. Considering antimicrobial resistance, four individuals (30.8%) presented Gram-positive bacteria resistant to vancomycin and/or methicillin, and six (46.2%) were sensitive to both antibiotics.

Discussion

Recent technological developments have led to the proliferation of new and rapid diagnostic tests that hold promise for the improved management and control of infectious diseases. Rapid diagnostics help to determine earlier in a patient's presentation the need for antibiotics, when applicable, which has implications in treatment that may, in turn, change outcomes [3]. Here, more than 25 different pathogens were investigated, with HCV being the most commonly requested.

Seasonal variation of diseases has been studied for centuries. Temperature, humidity, ultraviolet radiation, flora, and fauna all change with seasons, and consequently cause a variation in the frequency of occurrence of infectious diseases [4]. Patients' distribution per year showed a higher number of patients in the fall months (October to December). One explanation for this observation is the fact that, in the São Miguel's population, leptospirosis and respiratory virus infections also occur more frequently in these months. Indeed, these were the diagnoses that presented seasonality, as expected. Another explanation is the transition from summer to the rainy season, which leads to higher host susceptibility due to weather change adjustments. Moreover, these changes also make the conditions favorable for the survival of pathogens causing infectious diseases.

Molecular diagnosis of leptospirosis

Leptospirosis is a worldwide zoonotic and a recognized neglected infectious disease that is caused by spirochetes of the *Leptospira* genus from the family *Leptospiraceae* [5]. These spirochetes are responsible for severe human disease leading to a systemic infection, characterized by clinical manifestations that vary greatly from flu-like symptoms to multiple organ failure and death [6]. Leptospirosis is known for its endemicity, and is considered a public health problem due to its high annual incidence rate in semi-tropical climates, such as the Azores Islands (Portugal) [5,6]. Transmission of leptospirosis can occur through direct contact with an infected animal or its urine, placental fluids, or milk. Infection can also occur through exposure to a contaminated environment and, in particular, to water containing *Leptospira* spp. Entry into the body can occur via abraded skin, mucous membranes, consumption of contaminated meat or water, and inhalation. Once the bacteria enter the body, there is an incubation period of about 10 days. They multiply and circulate throughout the blood system, causing an antibody response that generally clears bacteria from the body, except for certain sequestered sites (kidneys, eyes, urogenital tract, central nervous system) [7]. The data showed that males and farmers were the most affected by this pathogen. These results are in accordance with the study developed by Vieira *et al.* [5] and Esteves *et al*. [6], where a significant link to professional activities and/or contact with rodents and domestic animals (natural reservoirs for leptospires) was observed.

Seasonal variation in occurrence is a common feature of infectious diseases. Here, the results demonstrated a seasonal winter distribution of leptospirosis. This seasonality is probably associated with the Azores climate conditions that are favorable for *Leptospira* spp. transmission, such as high levels of humidity (77.0% average/year) and precipitation. In fact, this same trend was previously observed by Vieira *et al.* [5].

The incidence of any disease is essential information for long-term planning of health care. The mean annual incidence of leptospirosis (32.9/100,000) was much higher than that previously reported for the Azores (11.1/100,000) [5]. Overall, the Azores stands out when compared with mainland Portugal (2.0/100,000) and the Madeira archipelago

(1.8/100,000). These observations may be explained by the fact that in the hospital, the availability of a molecular test with a timely response to clinicians (about six hours) enhanced the awareness about this disease. This increased the number of clinical requests and, consequently, the number of molecularly confirmed mild to severe cases. In fact, a direct consequence of the implementation of this molecular test is the absence, since 2007, of reported deaths caused by leptospirosis on the São Miguel Island (Maia DF, Mota FM and Paiva C, personal communication). Overall, these results validate the relevance of leptospirosis early detection, by PCR-based methods, in terms of Azorean public health.

The molecular test implemented for leptospirosis is performed directly on serum and urine samples of the same patient, without the need for bacterial culture. This represents a very important advantage in the understanding of the infection's progression. In fact, the DNA detection of *Leptospira* spp. on the patient's serum is indicative of an early stage of infection, whereas detection in the patient's urine (leptospiruria) usually occurs during the second week of infection, being indicative of a more advanced stage of infection [8]. The results demonstrated a higher number of positive results in urine (18.2%) compared with serum (10.7%). This observation may be explained by the fact that, since leptospirosis symptoms are similar to those of the flu, patients seek medical attention when symptoms worsen, which occurs at a later phase of the disease.

Molecular diagnosis of tuberculosis

Tuberculosis continues to be one of the most devastating and widespread infections in the world. Molecular diagnosis of tuberculosis has enabled rapid detection of MTB in clinical specimens, identification of mycobacterial species, and detection of drug resistance, which facilitates appropriate treatment initiation [9]. Overall, the mean annual incidence of TB was 4.7/100,000, which is lower when compared with the incidence in 2012 for mainland Portugal (21.6/100,000) and the Azores archipelago (6.5/100,000) [10]. This observation is explained by the fact that, according with the international guidelines for tuberculosis care [11], the current standard for TB diagnosis is a positive microbacterial culture from a body-fluid or tissue sample. Moreover, molecular testing is performed only when clinical symptoms justify such testing. For example, it is performed on patients suspected of having pulmonary tuberculosis whose sputum smears are negative [12]. Our lab, which only performs molecular techniques, does not have access to all individuals suspected of having TB. This fact makes it difficult to have more concise data on TB epidemiology for the São Miguel Island population. Nevertheless, it would be interesting to develop a population-based retrospective and prospective research project on TB epidemiology, involving clinical as well as conventional and molecular microbiology methods.

Men seem to be more affected by tuberculosis than women, with a male/female ratio of 1.9 ± 0.6 for the worldwide case notification rate [13]. Here, gender distribution showed a higher proportion of males (61.5%) affected by MTB. These results are in agreement with those presented by the Portuguese National Plan to Fight Tuberculosis [10], where, in 2012, the majority of TB cases were in males (65%) with an age range of 35–54 years.

Drug-resistant TB is of great importance worldwide as it is a good indicator of the control of TB in the population, and it also makes therapy more expensive and more difficult to implement [14]. Resistance to rifampicin (RIF) results showed that eight individuals presented MTB sensitive to rifampicin. Where mono-resistance to isoniazid is common, mono-resistance to RIF is rare. RIF resistance occurs most often in strains that are also resistant to isoniazid, making it a surrogate marker for multidrug-resistant tuberculosis (MDR-TB) [14]. In fact, the data demonstrated that of the four isolates resistant to RIF, only one was sensitive to isoniazid.

Molecular diagnosis of hepatitis B and C virus

Viral hepatitis is a major public health problem and is caused by infection with any of at least five distinct viruses: hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV). The morbidity and mortality in the human population from both acute infection and chronic sequelae is associated with this disease [15]. Here, a total of 149 individuals (59.4%) had HCV, 12 (4.8%) had HBV, and two had HBV/HCV coinfections.

In mainland Portugal, around 1% of the population is infected with HBV [16]. These numbers are not known for the São Miguel Island; however, 46.2% tested positive, with the majority being between 25 to 44 years of age. These results are in agreement with the ones reported by the Viral Hepatitis Prevention Board (VHPB) [17]. In terms of gender, the data showed equal distribution among males and females. According to VHPB, in mainland Portugal, with respect to hospital

admissions in 2004–2008, there was a male/female ratio of 2.1/1. This is not observed in the present study; nevertheless, the small sample size must be taken into consideration. Concerning HBV genotypes, genotype D was the most frequent, followed by genotypes A and E. This observation is in agreement with the known geographic distribution pattern for this virus, since genotype D is more common in Western Europe [18].

HCV is the most common infectious cause of chronic liver disease in Europe. Worldwide, approximately 3% of the population is estimated to be infected, which corresponds to around 200 million people at risk of developing serious liver-related morbidity [19]. In this study, 149 individuals tested positive for this virus, and a clear bias towards males was observed. These results are in accordance with those of Barra *et al.* [20], where 65% of infected individuals were males.

Reinfection is defined as a case in which an initial infection is completely resolved prior to a subsequent infection [21]. This can be either a reinfection with a different genotype/subtype than to the initial infection, or with the same subtype but a different strain. Two individuals showed infection by two different HCV viruses in two different time points. Based on clinical data, we were unable to confirm a reinfection due to re-exposure since we could not determine if the patients had cleared infection upon treatment.

HCV coinfection has been documented in individuals with ongoing risk behaviors, with the phenomena being detected in 2%–10% [21]. We identified coinfection in five patients (3.4%), all with risk behaviors, namely injection-drug use.

HCV genotypes are known to have a distinct geographic pattern of distribution and have been used to trace migration of populations from geographically distant regions. According to Velosa *et al*. [22], in mainland Portugal, the genotype 1 is the most frequent (60.0%), followed by genotypes 3 (25.0%), 4 (9.0%), and 2 (2.0%). The same trends were observed in our results, where genotype 1 was the most common (62.4%). Considering genotype 1 sub-typing, data showed a predominance of genotype 1a (61.1%). These results are not in agreement with those presented by the VHPB [17], where, in mainland Portugal, there was an increased frequency of genotype 1b (34%–59%). This observation validates the importance of conducting epidemiological studies in different regions of country, since information on HCV prevalence, viremia, and genotypes is critical for developing strategies to manage or eliminate HCV infection.

The vertical HCV transmission rate is estimated to be about 5%, ranging from 3% to 10% [23]. Mother-to-child transmission of HCV analysis showed that only one (7.7%) infant presented the virus. Benova *et al.* [24] observed that the risk of HCV vertical transmission among children born to HIV-positive women was more than double compared with those born to HIV-negative women. The biological mother of the positive case was HIV-positive.

Molecular diagnosis of herpesvirus family

Of the more than 100 known herpesviruses, eight routinely infect only humans: herpes simplex virus types 1 and 2 (HSV1, HSV2), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus 6 (HHV6), human herpesvirus 7 (HHV7), and human herpesvirus 8 (HHV8). These are a clinically important group of viruses that can cause a variety of diseases [25]. In the present study, a total of 199 individuals were tested for the presence of herpesvirus nucleic acids, the majority (85%) being negative.

Cytomegalovirus is a ubiquitous pathogen that infects 40% to 90% of adult human populations [26]. The results showed that the majority of CMV infected individuals (6 out of 10) were elderly. According to Bennet *et al.* [27], CMV seropositivity may contribute to negative health effects in more elderly adults due to elevated inflammation, which increases with age and is associated with many chronic diseases prevalent among elderly adults. Oppositely, CMV infections in neonates may cause severe sequelae, including sensorineural hearing loss, cerebral palsy, microcephaly, cognitive impairments, and mental retardation [28]. Here, one child was positive (5.5%). His mother seroconverted in the second trimester of pregnancy. CMV infections are often asymptomatic in healthy individuals, but can cause severe organ and life-threatening diseases in immunocompromised patients [26]. In the present study, three patients were monitored to prevent a CMV infection after organ transplantation. None presented any positive test results for the virus.

Herpes simplex virus – HSV1 and HSV2 – infections are highly prevalent worldwide and are characterized by establishing lifelong infection with periods of latency interspersed with periodic episodes of reactivation [29]. Only two individuals were HSV1 positive. In these two patients, the samples analyzed were skin lesions. These viruses commonly cause recurrent infection affecting the skin, mouth, lips, eyes, and genitals. Although less common, HSV1/2 also cause potentially deadly illnesses, such as herpes

encephalitis, erythema multiforme, eczema herpeticum, neonatal herpes, and disseminated herpes [30]. These viruses were evaluated in combination with other infectious agents, for differential diagnosis, in cerebrospinal fluid and serum samples. In these, only three individuals had HSV1; all were > 63 years of age and immunologically compromised.

Molecular diagnosis of respiratory virus

The viruses most frequently involved in acute respiratory infections are adenoviruses, influenza viruses, parainfluenza viruses, respiratory syncytial viruses, and rhinoviruses [31]. Here, six individuals were infected by influenza virus, three with influenza A virus, and three with influenza B virus. According to the Portuguese National Influenza Reference Laboratory report [32], these viruses were in circulation during the 2012/2013 influenza season. In temperate climate regions, influenza epidemics display a distinct seasonality with widespread infection, typically occurring during the winter season months. We observed positive influenza cases in the winter and spring months, which is in accordance with the flu season in Portugal. Nevertheless, the small number of infected individuals does not allow for a more precise determination of the case distribution pattern.

Molecular diagnosis of sepsis

Sepsis is the body's overwhelming and life-threatening response to an infection, which can lead to tissue damage, organ failure, and death (http://www.sepsis.org/). Overall, in the 13 individuals molecularly tested, we identified a pathogenic agent, the majority of the time in a co-infection situation. According to Sakr *et al*. [33], female patients were less likely to have severe sepsis and septic shock on admission to the intensive care unit (ICU) compared to male patients, who presented a younger age. In the present study, males and females were equally affected; however, females were older than males. In Portugal, around 22% of ICU admissions are due to sepsis [34]. Here, the majority of patients were inpatients of the ICU.

Antimicrobial resistance is a major concern of public health, since infections with multidrug-resistant microorganisms are associated with increased risk of complications, higher hospitalization rates, increased healthcare costs, loss of productivity, and increased mortality [35]. The results showed that four individuals (30.8%) presented Gram-positive bacteria resistant to vancomycin and/or methicillin, and six (46.2%) were sensitive to both antibiotics. Considering that the Magicplex sepsis real-time test (Seoul, Korea) identifies only the Gram-positive bacteria genus, we were unable to determine which microorganisms were resistant to these antibiotics.

Conclusions

Significant progress has been made in the diagnosis and timely management of infectious diseases, reducing their impact in the world. The present study demonstrated that each type of pathogen affects different age groups, genders and occupations, due to different types of exposure to the pathogen and the pathogen's form of transmission. A good example of this is the observation that males and farmers were more affected by leptospirosis than were others. Overall, the present work constitutes a valuable descriptive analysis of molecular diagnosis of infectious diseases in the São Miguel Island (Azores, Portugal) population and contributes to the knowledge of the epidemiology of infectious diseases on the island.

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Authors' contributions

AS and RMM contributed equally to this work. AS, RMM and CCB performed the experiments, statistical analysis, and drafted the first version of the manuscript. TP, MJB, SB and RC carried out molecular diagnostics tests. CCB and LMV provided scientific supervision, as well as draft and revision of the manuscript. All authors read and approved the final manuscript.

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Annex – Supplementary Items

Supplementary Table 1. Molecular assays used for detection and identification of infectious pathogens.

Pathogens (ID)	Diagnoses assays		
	Designation	**Reference (company)**	**LOD[1]**
Bacteriology			
Leptospira spp. (A1)	In-house	Int J Infect Dis 14: e148-e153[2]	In-house
Mycobacterium tuberculosis and rifampicin resistance (A2)	Xpert MTB/RIF	GXMTB/RIF-10 (Cepheid)	131 CFU/mL
Virology			
Hepatitis C virus (B1)	Genotyping: AmpliSens HCV-genotype-FRT PCR kit	TR-V1-G-2x(RG,iQ,SC)-CE (Ecoli Ltd)	1,000 IU/mL
	Detection: Quantification of Hepatitis C Virus Advanced kit	Path-HCV (PrimerDesign Ltd)	< 100 copies of target sequence
Hepatitis B virus (B2)	Genotyping:		
	In-house or	J Clin Microbiol 48: 1105-1111[3]	In-house
	INNO-LiPA HBV Genotyping	80691 (INNOGENETICS)	Not specified
	Detection: Quantification of Hepatitis B Virus Advanced kit	Path-HBV (PrimerDesign Ltd)	< 100 copies of target sequence
Cytomegalovirus (B3)	CMV Real-TM Quant	V7-100/2FRT (Sacace)	500 copies/µL
	SmartCMV	XSQCMV-100N-032 (Cepheid)	357 copies/mL
Herpes simplex virus (B4)	SmartCycler HSV1/2 Reagents Primer and Probe Set	SCHSV1/2-CE-40 (Cepheid)	17.91/23.38 geq/rxn
Epstein-Barr virus (B6)	Smart EBV	XSQ EBV-100N-032 (Cepheid)	381 copies/mL
Influenza virus (B9)	Xpert Flu	GXFLU-CE-10 (Cepheid)	Not specified
Respiratory virus panel	Magicplex RV Panel Amplification	RV8000Y (Seegene)	Not specified
Enterovirus (B10)	Smart EV	SCEV-CE-40 (Cepheid)	Varies from 0.178 to 11.4 TCID (50)/mL, depending virus
Adenovirus (B11)	RealCycler Adenovirus	ADNV v.5 (Progenie Molecular)	10 copies/mL
Sepsis			
Sepsis (C1)	Magicplex Sepsis Real-time Test	SE8000Y (Seegene)	30 CFU/mL

[1] LOD: lower limit detection; [2] Gonçalves AT, Paiva C, Melo-Mota F, Vieira ML, Carreira T, Nunes MS, Mota-Vieira L, Ahmed A, Harstkeerl RA, Hyde K, Collares-Pereira M (2010) First isolation of human Leptospira strains, Azores, Portugal. Int J Infect Dis 14: e148-e153; [3] Malmström S, Berglin-Enquist I, Lindh M. Novel Method for Genotyping Hepatitis B Virus on the Basis of TaqMan Real-Time PCR (2010) J Clin Microbiol 48: 1105-1111.