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UNIVERSITÀ DEGLI STUDI DI VERONA

DIPARTIMENTO DI PATOLOGIA

DOTTORATO DI RICERCA IN
BIOMEDICINA TRASLAZIONALE

CICLO XXII

TESI DI DOTTORATO

HIV-1/2 PREVALENCE AMONG MSM (MEN WHO HAVE SEX WITH MEN) IN EASTERN AND SOUTHERN EUROPE: NON INVASIVE ORAL FLUID TESTING AS A TOOL FOR SURVEILLANCE AMONG HARD-TO-REACH GROUPS (REFERENCE LAB FOR EUROPEAN PROJECT SIALON P.N. 101046).

S.S.D. MED/04

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1.1 Global HIV estimates

HIV remains a global health problem of unprecedented dimensions. Despite prevention efforts, the HIV/AIDS pandemic continues to expand globally at the rate of 13,000 new infections/day, showing no sign of slowing, particularly in low- and middle-income countries, where about 95% of infected persons live.

According to the World Health Organization (WHO) and the Joint United Nations Programme on HIV/AIDS (UNAIDS), a total of 33.4 million [31.1-35.8 million] people, including 2.1 million children [1.2-2.9 million] were estimated to be living with HIV/AIDS through the end of 2008, with a prevalence threefold higher than 1990 (Table I) [1].

As regards new infections, they reached more than 2.7 million [2.4-3.0 million] in 2008 with 430,000 [240,000-610,000] children less than 15 years. HIV-related deaths were 2 million.

Table I: HIV/AIDS global estimates in 2008.

People living with HIV/AIDS		
Adults	Male	15.6 million
	Female	15.7 million
Children < 15 years		2.1 million
Total		**33.4 million**
New HIV infection in 2008		
Adults		2.3 million
Children < 15 years		430,000
Total		**2.7 million**
Deaths from AIDS in 2008		
Adults		1.7 million
Children < 15 years		280,000
Total		**2.0 million**

Unknown 27 years ago, HIV has already caused an estimated 25 million death worldwide. HIV is among the leading causes of death worldwide and it causes more deaths than any other infectious disease. Around 95% of HIV infected people live in non-industrialised countries with not sufficient financial resources to deal with the HIV/AIDS epidemic. It is estimated that the vast majority of AIDS deaths are due to inadequate access to HIV prevention and treatment [2].

Heterosexual transmission is now the dominant mode of transmission and accounts for more than two-third of all HIV infections worldwide, although transmission in IDU (injecting drug users) and MSM (men who have sex with men) constitute a significant fraction of infections (10% and 5-10%, respectively) [1,3]. Mother-to-child transmission of HIV continues to occur at significant rates in the developing world, accounting for roughly 400,000 new infections annually [2,4,5].

Transmission through injection drug use appears to play a major role of HIV epidemics in several region of the world, particularly Eastern Europe and Asia [6]. In developed countries, the interplay between increased high risk sexual behaviour and injecting drug use fuels HIV epidemics.

The global epidemic is not homogenous within regions (Fig.1).

Sub-Saharan Africa remains the region most heavily affected by HIV infection: home to just over 10% of the world population, it accounts for more than two thirds (22.7 million) of the global number of infected people and for 72% (1.4 million) of all AIDS deaths [1,2]. Moreover, 90% of all children affected worldwide live in that region. Within the region, South-Africa is the worst

affected country, with more than 5.7 million people living with HIV. The leading mode of HIV transmission in Sub-Saharan Africa is through heterosexual route.

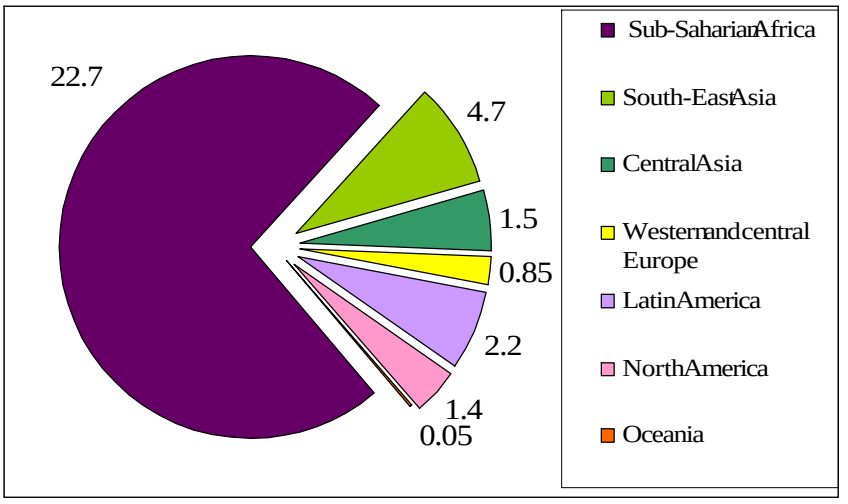


Fig.1 Global adult HIV/AIDS prevalence by region in 2008 (million people).

Asia is becoming the epicentre of second largest epidemic with 350,000 new infections annually and an estimated 4.7 million people living with HIV at the end of 2008 [1].

In Central Asia and in countries of the former Soviet Union, the HIV epidemic has increased faster between 2000 and 2006 (20-fold increase) than in any other area of the world, reaching an estimated 1.5 million HIV cases at the end of 2008. Nearly 90% of the HIV cases were reported from the Russian Federation and from Ukraine (infection rate: over 200/million) [7,8]. The vast majority of them were found among people below the age of 30 years (by comparison, in Western Europe some 30% of people with HIV fall in that age group). HIV epidemic in the region is driven by injecting drug use and increasingly by heterosexual transmission.

An estimated 2.3 million people are living with HIV in North America and in Western and Central Europe. In high-income countries, where the great majority of people who need antiretroviral treatment have access to it, people living with HIV are surviving longer than infected people elsewhere [1].

In the United States, throughout the 1980s, the number of new AIDS cases increased rapidly. With the advent of HAART in 1995, there was a step decline in the number of new AIDS cases and deaths. Since 2000, however the decline in the number of persons who progress to AIDS each year has levelled off.

Currently, an estimated 1.1 million individuals in the United States are living with HIV infection, one-fifth of whom are unaware of their infection and thus, miss the opportunity to receive life-saving antiretroviral therapy [9,10].

Despite the substantial reductions in deaths and AIDS diagnoses since the mid-1990s, the US epidemic continues, with an estimated 50,000 new HIV infections occurring each year, many of which occur in MSM and ethnic minority populations [11].

The largest proportion of newly diagnosed HIV cases was among homosexual males (almost half cases), followed by infections due to high-risk heterosexual contact (about 30%), to injection drug use (22%) and due to a combination of homosexual transmission and injection drug use (5%) [11-13].

In Latin America, an estimated 170,000 new HIV infections occurred in the 2008, bringing the number of people living with HIV to an estimated 2 million [1,2]. HIV trends in Latin America have also been relatively stable in the past decade, with most transmission attributed to MSM and heterosexual intercourses.

MSM account for the majority of new HIV infections in Australia and New Zealand [1].

1.2 European Union HIV estimates

According to UNAIDS estimates, the adult HIV prevalence in the European Community (EC) was 0.3% in 2007. It was generally lower in Northern countries such as Sweden and Finland (0.1%) than in Southern countries such as Portugal (0.5%), Spain (0.5%) and Italy (0.4%). Around 850,000 people were estimated to be living with HIV in the European Union at the end of 2008 [1,7].

HIV epidemic continues to occur at substantial rates in EC with evidence of an increase of new diagnoses from 2000 to 2008 (42 to 61 cases per million). About 26,000 newly diagnosed cases of HIV infection were reported by the countries of the EU in 2008, with the highest rates being reported from Estonia (400/million), Latvia (158/million) and United Kingdom (119/million) [14].

Rates of HIV diagnosed cases have doubled also in Bulgaria, Czech Republic, Hungary, the Netherlands, Slovakia and Slovenia; rates have increased by more than 50% in Germany, Norway, Lithuania and United Kingdom.

After falling dramatically in the late 1990s, following the introduction and widespread use of combination antiretroviral treatment, AIDS incidence in the EU countries has decline steadily. Thus, the number of reported AIDS cases has decreased by more than 50% between 2000 and 2008 [7].

EU countries with the highest rates of AIDS diagnosis are Estonia (46/million), Latvia (44/million), Portugal (36/million) and Spain (29/million).

In EU countries the proportion of heterosexually acquired cases of infection exceeds 50% of the total, whereas over a third of new HIV cases have been reported among MSM (reaching 50% of cases in Czech Republic, Hungary, Slovakia and Slovenia) and about 10% of cases are seen among IDU (the largest transmission group being in Poland, Estonia and Lithuania) [7,15,16].

1.3 HIV estimates in Italy

In Italy, to obtain reliable data on the HIV epidemic, local surveillance systems of the new HIV diagnoses have been set up since 1988 in some regions and provinces (Piemonte, Liguria, Friuli Venezia-Giulia, Veneto, Puglia, Emilia-Romagna, Lazio, Trento, Bolzano, Sassari, Catania). After a rapid spread of HIV infection in the 1980s, there has been a progressive decrease in the number of new diagnoses during 1990s; in more recent years this trend has levelled-off [17-20].

In 2008, an estimated 4,000 people (6.7 per 100,000 inhabitants) became newly infected with HIV in Italy, bringing to 170,000-180,000 the number of individuals living with HIV. About 60% of newly diagnosed patients have AIDS [21].

The mean age of the newly infected people is 34 ys in women and 38 ys in men. The incidence is raising among immigrants, where one third of the total number of new infections occurs [22].

AIDS incidence in Italy is estimated to be of 20 cases/million. Since the AIDS mortality peaked in 1995 reaching 4,500 deaths per year, there has been a progressive decline in mortality rate (147 deaths in 2008 vs. 588 deaths in 2004) due to effective HAART (Highly Active Antiretroviral Therapy) [7].

The epidemiology of HIV infection has greatly changed over time since 1988, when injecting drug users were driving the epidemic. Among adults diagnosed with AIDS in Italy, in the last decade there has been an increase in heterosexual (from 25% in 1998 to 44% in 2008) and homosexual (from 17% to 24%) transmission route, where as the transmission through the injection drug users has decreased by half (from 48% to 22%) [21,23].

1.4 HIV estimates among MSM

As in the beginning of the epidemics, consistently high levels of HIV infection have been found among MSM worldwide, with more than 200,000 people becoming newly infected each year [1,24,25].

MSM include a variety of subsets, ranging from homosexual to bisexual, transgender and self-identified heterosexual men who engage in sex with other men. They represent a high risk population, contributing significantly to the spreading of HIV infection [26].

In much of the high income countries, HIV transmission among MSM is the main driver of the epidemics.

In the United States, MSM represent the risk group most severely affected by HIV, accounting for nearly half HIV cases (500,000 people infected with HIV infection). Additionally, this is the only risk group in the U.S in which the annual number of new HIV infections is steadily increasing since the early 1990s, with an estimated 29,000 newly-diagnosed HIV infections [27].

In their most recent account, the European surveillance network, EuroHIV, reported that MSM constituted 40% (about 8,300 cases; 60 per million men aged

15-64 years) of all newly diagnosed HIV infections in EU countries in 2008. In EU countries, the number of HIV cases identified among MSM increased by 40% between 2003-2008, thus suggesting a resurging epidemic involving this high risk population [7,28]. The highest rate was reported in United Kingdom (130/million male population), where HIV cases rose by 74% between 2000 and 2007, and the lowest in Romania (1/million men) [29]. In the Netherlands, Hungary, Czech Republic, Slovakia and Slovenia, the total number of new reported HIV cases in MSM represented more than 50% of all new reported cases [26].

In former Soviet Union countries, less than 1% of new HIV infections were in MSM (about 120 cases) in 2008 [7,26]. HIV prevalence rates among MSM were estimated to be 5% in Georgia, 6% in Russian Federation and have reached as high as 10-23% in Ukraine [1,25,26].

Lack of awareness of HIV infection status is a likely reason for continuing high-risk behaviours in MSM. The using of alcohol and illegal drugs (substance abuse), appears to contribute to increased risk for HIV infection and other STDs (sexually transmitted diseases).

Current guidelines recommend that screening be performed at least annually in high risk groups, such as MSM [30,31].

2. STRUCTURE AND BIOLOGY OF HIV VIRUSES

2.1 Structure and genomic organization of HIV-1/2

The human immunodeficiency viruses (HIV-1 and HIV-2) are members of the Lentivirus genus of the *Retroviridae* family, commonly called retroviruses [32-34].

The HIV genome contains the *gag*, *pol*, and *env* genes which encode structural proteins, viral enzymes, and envelope glycoproteins, respectively (Table II).

The *gag* gene encodes a precursor protein (p55) that is cleaved into four major structural proteins: p17, p24, p9 and p6.

Products of the *pol* gene include three enzymes: protease (p11), reverse transcriptase (p66 and p51) and integrase (p32). The protease is responsible for cleavage of *gag-pol* precursor polypeptides into functionally active proteins. The reverse transcriptase of HIV-1 is a RNA-dependent DNA polymerase responsible for replicating the RNA genome. The integrase is required for provirus integration into the host genome.

The *env* gene encodes a glycosylated polypeptide precursor gp160 that is cleaved to form the envelope surface glycoprotein (gp120) and the transmembrane glycoprotein (gp41).

In addition, HIV genome contains at least six short regulatory sequences involved in the activation of HIV transcription and in the modulation of viral gene expression (*tat, rev, nef, vif, vpr, vpu*) [35]. They encode auxiliary or regulatory proteins, including Trans-acting transcriptional activator- *tat*-, the Regulator of

viral expression-*rev*-, Negative effector -*nef*-, Viral infectivity factor -*vif*-, Viral protein U -*vpu*- and Viral protein R -*vrp*-.

The *tat* protein up-regulates HIV expression at transcriptional and post-transcriptional level. The *rev* protein promotes nuclear export of unspliced or partially spliced viral mRNAs encoding the viral structural proteins. The *vif* gene encodes a protein that suppresses the antiretroviral effect of the cellular enzyme APOBEC, preventing viral DNA degradation. The *vpu* gene encodes a protein that promotes the CD4 degradation and influences virion release. The *vpr* gene encodes a protein that is involved in nuclear translocation of viral genome and facilitates virus production by arresting infected cells in G2 of the cell cycle. The *nef* gene encodes a protein that promotes down-regulation of cell-surface-bound CD4 (receptors) and cell surface expression of MHC I.

Nef is one of the first HIV proteins to be produced in infected cells and is the most immunogenic of the accessory proteins.

These genes are flanked by long terminal repeat (LTR) elements, that direct and regulate the expression of the viral genome.

HIV is an icosahedral virus 100 to 120 nm in diameter, provided with two identical copies of viral genomic RNA condensed in a nucleocapsid core.

The nucleocapsid core is cone-shaped and consists of two identical copies of single-stranded RNA approximately 9.2kb in length, strictly linked to the nucleocapsid protein –p9– (NC) and surrounded by the structural capsid proteins –p24–.

The viral envelope is derived from the outer limiting membrane of the host cell as the virus buds from infected cells. Inserted in the envelope are the envelope glycoprotein spikes, each consisting of a complex of three Env gp120-gp41 heterodimers formed by the two major envelope proteins, the external gp120 and the transmembrane gp41. Underlying the envelope is the viral matrix protein p17.

Biologically, HIV-2 is structurally analogous to HIV-1: the HIV-2 genome exhibits about 40% to 50% homology in overall nucleotide sequence. Some of its protein components differ, most notably the envelope glycoproteins gp105 and gp36, as well as the core protein p16 and p26 or the *pol* protein p68 and p34.

The genome of HIV-2 lacks the *vpu* gene and exhibits the presence of *vpx* gene, which is a homolog of HIV-1 *vpr* [36] (Table II).

Table II: Major HIV-1/2 gene products.

Gene	HIV-1	HIV-2	Protein/Function
Gag	**P24**	**P26**	Capsid protein
	P17	**P16**	Matrix protein
	P9	P9	RNA binding protein
	P6	P6	RNA binding protein
Pol	**P66/p51**	**P68**	Reverse transcriptase (RT)
	P11	**P14**	Protease / post-translational processing of viral proteins
	P32	**P34**	Endonuclease and ligase / viral DNA integration into cell host genome
Env	**Gp120**	**Gp105**	Envelope surface glycoprotein
	Gp41	**Gp36**	Envelope transmembrane protein
Tat	**P14**	P14	Regulatory protein / transcription of viral genes
Rev	**P9**	P9	Regulatory protein / viral mRNA transport out of the cell nucleus
Nef	**P27**	P27	Accessory protein / decrease in level of CD4 receptor on cell surface
Vif	**P23**	P23	Accessory protein / increase in virus infectivity
Vpr	**P15**	**P15 [Vpx]**	Accessory protein / promotion of virus replication
Vpu	**P16**	-	Accessory protein / release of new virions

The main antigenic differences between HIV-1 and HIV-2 are in the envelope glycoproteins which are responsible for much of the reactivity with antibodies. By contrast, antibodies to the *gag* and the *pol* proteins generally cross-react.

Modes of transmission of HIV-2 are the same as for HIV-1 but it is less transmissible both sexually and perinatally. The frequency of transmission is reduced, probably due to a lower viral burden during the relatively long asymptomatic period of the disease [36].

2.2 HIV genotypes

The HIV epidemic appears to be the result of zoonotic virus transmission from non-human primates to human in Africa [37,38].

Two genetically distinct viral types of HIV have been identified: HIV type 1 (HIV-1), discovered in 1983 and responsible for the worldwide AIDS pandemic, and HIV type 2 (HIV-2), first isolated in 1986 and endemic primarily in areas of West Africa (e.g. Guinea-Bissau and the Ivory Coast) and India. HIV-2, endemic in West African nations, has reached its highest prevalence of 8-10% in parts of Guinea-Bissau [32,39].

Sporadic outbreaks of HIV-2 infection have been reported outside Africa in European countries with historical links with west Africa such as France, the United Kingdom and Portugal, and also in South America [36,40-42].

The two distinct types of human immunodeficiency viruses (HIV-1 and HIV-2) have closely related counterpart in African primates: the chimpanzee subspecies *Pan troglodytes troglodytes* in West-central Africa have been identified as the natural reservoir of the HIV-1 pandemic and the sooty mangabeys in Western Africa as the source of HIV-2.

Due to this variability, phylogenetic sequence analyses of HIV-1 strains have identified three distinct groups: group M (major), which is responsible for most of the infections in the world; group O (outlier), a relatively rare viral form found originally in Cameroon and Gabon; group N, identified in only a limited number of people from Cameroon [43,44].

Group M, which is responsible for the vast majority (more than 95%) of HIV-1 infections worldwide, comprises 9 subtypes, or clades (A-D; F-H; J and K), as well as a growing number of major and minor circulating recombinant forms (at least 43), ranging from highly prevalent forms, such as CRF01_AE virus to a large number of CFRs that are relatively rare [45-47].

Subtype A viruses finds their highest concentration in areas of Central Asia, Eastern Africa and in Eastern European countries formerly constituting the Soviet Union. Subtype B dominates epidemics in Western and Central Europe, in the United States and Australia and is also common in several countries of Southeast Asia, Northern Africa, and the Middle East and among South African and Russian homosexual men.

Subtype C viruses is the overwhelming strain in Southern Africa, China and India.

Certain HIV-1 isolates appear to be recombinant, containing sequences from more than one subtype. Many countries have co-circulating viral subtypes that are giving rise to new CFRs.

The relevance of circulating recombinant form (CRFs) in the global HIV-1 pandemic is increasingly recognized, accounting for 18% of infections, and

representing the predominant local form in Southeast Asia (CRF01_AE) and in West and West Central Africa (CRF02_AG).

It is interesting to note that most new HIV-1 infections occur in developing countries, such as in Sub-Saharan Africa where non-B subtypes are prevalent, and then spreaded to industrialized countries through population movements from endemic countries [48].

HIV-2 variants are classified into eight major phylogenetic groups designed A through G, of which A and B are the majority strains.

These HIV-1 and HIV-2 groups and HIV-1 subtypes are now distributed throughout the world; all are found in Africa. HIV-1 clade C is becoming the most prevalent transmitted virus [43].

2.3 Replication cycle of HIV

The replication of HIV begins with the sequential high-affinity binding of the gp120 protein to the target cell associated-CD4 molecule, followed by binding to a chemokine co-receptor (either CCR5 or CXCR4), which culminates in a gp41-mediated fusion event and release of the viral core into the cell [49].

The CD4 molecule is a surface protein that marks the T-helper subset of T lymphocytes and is also expressed on the surface of monocytes/macrophages and dendritic/Langerhans cells.

Viruses able to utilize CCR5 can infect both macrophages and $CD4^+$ T cells, while viruses that only utilizes CXCR4 usually infect only $CD4^+$ T cells.

RNA H activity and DNA polimerase generate a double-stranded DNA copy of the viral RNA able to migrate to the nucleus and integrate within the host cell

genome, leading to the formation of the provirus [50]. Noteworthy, reverse transcriptase is an error-prone enzyme that generates multiple mutations at every cycle, thereby allowing the development of drug resistance and escape from immune surveillance.

At this point of the replication, the viral genome is vulnerable to cellular factors belonging to the APOBEC family, that can block the progression of infection this proteins bind to nascent reverse transcripts and deaminate viral cytidine, causing hypermutation of HIV genomes. HIV has evolved a powerful strategy to protect itself from APOBEC, through protein *vif*-mediated proteosomal degradation of APOBEC. In its DNA form, viral genome is inserted into the host genome.

Integrated DNA is transcribed into RNA by the host RNA polymerase II. Newly synthesized RNA can be multiply spliced to generate small transcripts that encode *tat, rev* or *nef,* in alternative can be singly spliced to express *env, gag* and *pol*, or remain unspliced and used as mRNA for *gag, pol* and *env*. Singly spliced and unspliced RNAs can be exported into the cytoplasm thanks to *rev* accessory protein.

Transcribed viral mRNA is translated into viral proteins, that allow the assembling of the new virions at the cell surface, along with newly synthesized viral RNA. Mature virions are released ready to infect new cells and begin the replication cycle once again. The entire process is extremely active, with $10^{8\text{-}10}$ viral particles produced each day. The proviral form may remain transcriptionally inactive or it may manifest varying levels of gene expression, up to active production of virus. Activation of HIV expression from the latent state causes the

transcription of the integrated proviral RNA into genomic mRNA and its translation into proteins.

3. CLINICAL AND IMMUNOLOGICAL CORRELATES OF HIV INFECTION

3.1 Time-Course of HIV infection

The natural history of HIV infection can be divided into three phases: primary infection with a transient acute retroviral syndrome -ARS-, followed by a period of clinical latency and finally, symptomatic disease culminating in AIDS [51].

Primary infection, which covers the period from the onset of infection until seroconversion, is characterized by rapidly increasing high levels of HIV replication together with significant reduction of HIV-specific $CD4^+$ T lymphocytes.

It is estimated that 50-70% of individuals with primary HIV infection experience an acute retroviral syndrome within 3-6 weeks after the onset of infection.

The clinical symptoms of acute HIV infection include flu-like or infectious mononucleosis-like symptoms, such as fever, pharyngitis, lymphadenopathy and maculo-papular skin rash, typically lasting 5-10 days. Severe illness, particularly acute encephalopathy, is occasionally found and is associated with poor prognosis.

Symptoms in the acute HIV syndrome occur along with a burst of plasma viremia, reaching levels up to 100 million copies of HIV-1 RNA per ml of plasma and gradually subside as an immune response to HIV, namely seroconversion, develops, as a result of the emergence of host antiviral immune response.

Both cell-mediated (production of HIV-specific cytotoxic lymphocytes T) and humoral (production of complement fixing and neutralizing HIV-specific antibodies) immune responses are responsible for the significant decrease of plasma viral load. Although the immune response succeeds in down regulating the viremia, HIV replication is never completely abrogated and reach a steady state, known as set-point, that is an important predictor of disease progression. High set-points are prognostic of the risk for faster course until the development of AIDS and death, while lower set-points are associated with a slower disease progression. Detection of acute HIV infection, where the virus infectiousness has been shown to be enhanced approximately 26-foulds than the later stages of the disease, is important for patients and for public health [52].

The clinical latency with no therapy may last up to 10 year or longer and occurs in the majority of patients. A small group of rapid progressors experiences AIDS within 1 to 2 years, whereas 5% to 10% of HIV long-term progressors remain symptom-free for longer than 20 years.

Active HIV replication is ongoing and progressive immunological impairment occur throughout the asymptomatic period. Widespread dissemination of the virus, with seeding of lymphoid organs and other tissue reservoirs results in depletion of $CD4^+$ cells in gut-associated lymphoreticular tissue and in lymph nodes, thereby leading to a long-lasting quantitative loss of $CD4^+$ helper T-cell function [53].

The continuous replication of HIV together with the elimination of host cells and chronic immune activation results over the years in loss in immune function and

increased susceptibility to infections and malignancies. The spectrum of clinical manifestations observed during the course of HIV infection changes as the $CD4^+$ T-cell count declines.

The most common clinical findings are represented by constitutional symptoms (fever, weight loss and diarrhoea lasting more than 1 month), persistent generalized lymphadenopathy (enlargement greater than 1 cm in diameter of two or more extrainguinal nodes of at least three months'duration), recurrent infections by Herpes Zoster virus in more than one dermatome, oropharyngeal candidiasis (thrush), recurrent vulvovaginal candidiasis, oral hairy leukoplakia by Epstein Barr virus [54,55].

A diagnosis of AIDS is made in anyone with HIV infection and a $CD4^+$ T-cell count less than 200 cells/μL, and in anyone with HIV infection who develops one of the HIV-associated diseases considered to be indicative of a severe defect in cell-mediated immunity [54,56].

Conditions listed in the 1993 AIDS surveillance case definition include [56]:

- $CD4^+$ T-cell count less than 200 cells/μL;
- opportunistic infections caused by viruses (retinitis, pneumonia, enteritis or encephalitis by Cytomegalovirus; chronic ulcers, pneumonia or esophagitis by Herpes simplex), bacteria (recurrent pneumonia by *H.influentiae* or *S. pneumoniae*), pulmonary and extra-pulmonary infections by *M. tuberculosis*, infections by *M. avium* complex -MAC- , recurrent *Salmonella* septicaemia), fungi (*Pneumocystis jiroveci* pneumonia, pulmonary and oesophageal candidiasis, disseminated cryptococcosis, coccidioidomycosis and

histoplasmosis) and protozoa (cerebral toxoplasmosis, chronic intestinal crysporidiosis and isosporiasis)

- neoplastic disease (Kaposi's sarcoma, lymphomas, invasive cervical cancer).
- HIV encephalopathy;
- progressive multifocal leukoencephalopathy;
- wasting syndrome (debilitating weight loss and diarrhoea).

Any of these conditions complicate the clinical course of the end stage of HIV infection and may cause death.

Clinical studies show that the HIV-2 disease progresses slower than in HIV-1 infection, resulting in a survival rate higher than that in the equivalent stage of HIV-1 infection with a mortality rate estimated to be two-thirds lower than that for HIV-1. However progression of disease in the later stages, as the immune response collapses, is fairly comparable between HIV-1 and HIV-2 [39,57-63] (Table III).

Table III: Clinical and immunological features of HIV-1/2 infection.

	HIV-1	**HIV-2**
Epidemiology		
World distribution	Pandemic	West Africa (prevalence: 1-2%)
Infection time-course		
Clinical evolution	Majority develop AIDS <2% : long-term non progressors	~20-25% develop AIDS remainder: long-term non progressors
Progression rate	Fast: 5-10ys in the majority	Slow: 10 ys or longer in the majority
Virus infectivity		
Viral load in blood	High	Low
Virus replication rate	Fast	Slow
CD4 receptor affinity	High (gp120)	Low (gp105)
Immunology		
Immune activation	Variable	Low
T-cell apoptosis	Increased	Lower than HIV-1
Decline in $CD4^+$count	Faster	Slow or none in asymptomatic
CTL responses	CTL escape mutants are common	Strong in asymptomatic
Neutralizing antibody	Less efficient, narrow specificity	Efficient, broad specificity

3.2 HIV virological and serological markers

Following exposure to HIV infection, HIV-specific markers appears in the blood in the following chronological order: HIV RNA, p24 antigen and anti-HIV antibodies [64].

Within the few first days of HIV infection, the virus starts an active replication in mucosal and lymphoid tissue that drains the inoculation site. This phase is referred to as the "eclipse phase" and usually lasts 10-14 days and occasionally up to 6 months.

Viral active replication leads to the appearance of infectious viremia, as measured by plasma viral RNA, within two weeks after infection [65].

Viral antigens become detectable in plasma soon after viral RNA. The protein most widely used in HIV diagnosis is the capsidic protein p24, which appears usually within the first two to three weeks after acute infection. Its presence in the serum is transient, and its disappearance coincides with the development of both humoral and cellular responses to infection.

The time period before antibody appears is referred to as serological "window" period and is characterized by seronegativity, detectable viremia (as measured by RNA or p24 antigen), and variable CD4 lymphocyte levels.

A transient immunoglobulin M (IgM) antibody response against the capsidic (p24) or envelope (gp160, gp120, gp41) proteins is usually the first to appear and is followed by a long-lasting immunoglobulin G (IgG) response. The appearance of IgG antibodies against the core (p24) and envelope (gp160,120, 41) proteins

are then followed by antibodies against HIV viral enzymes, a process called "seroconversion".

In most of infected persons, anti-HIV antibodies become detectable within three weeks, but in a small percentage of individuals (5%), seroconversion may require up to 6 months [65,66].

The appearance of antibody is clearly a major mechanism in decreasing viremia, as noted by the decrease in viral copies and p24 antigenemia, as antibody levels rise.

With disease progression resulting in a decline in immunocompetence, virus replication increases (HIV RNA and p24 antigen levels increase), anti-p24 levels decrease, $CD4^+$ cell count falls less than 200/μL and the syndrome of AIDS manifests [67].

4. STATE OF ART IN HIV TESTING

4.1 Overview of HIV diagnostic tests

The ability to diagnose HIV infection is a critical component in the efforts to decrease the ongoing spread of the epidemic, because HIV-serostatus awareness can help reducing risk behaviour associated with HIV transmission and provides life-saving therapy to the newly diagnosed patients [68].

Diagnostic assays for HIV infection have evolved substantially since the introduction of antibody testing in 1985 [66,69].

HIV infection is identified either by the detection of HIV-specific antibodies in serum or plasma or by demonstrating the presence of the virus (nucleic acid detection) [70].

Current routine laboratory diagnosis of HIV infection is mainly based on the detection of specific anti-HIV antibodies. The HIV testing algorithm comprises initial serological screening for anti-HIV antibodies by using conventional enzyme immunoassays (EIA) or rapid immunoassays, followed by confirmatory testing of reactive specimens with a supplemental test (Western Blot or indirect Immunofluorescence assay -IFA- or Line Immunoassay -LIA-).

Regardless of the type of screening method used, a specimen producing a positive result will be tested again with the same or a different screening test. If that sample is repeatedly reactive and independent sample of the patient test will be assayed with a confirmatory test. Screening assays are highly sensitive, but not optimal with regard to specificity, particularly in testing low risk populations;

therefore the sequential use of confirmatory tests, that have a high specificity, enhances the accuracy of HIV diagnostic testing [71-74].

4.1.1 *Screening tests for HIV detection*

The enzyme immunoassay (EIA) is the most commonly used type of test to screen for HIV infection because of its simple methodology, high sensitivity, and suitability for high-volume testing. EIAs use an HIV antigen coated on a microwell plate to detect any HIV antibodies present in the specimen [64,75].

Four generations of EIAs have been developed, with later formats having improved test performance and shorter antibody-negative window period [76,77].

The first generation assays, developed in 1985 for diagnosis of HIV infections, used viral protein lysates derived from whole disrupted virus, adsorbed to the solid phase for detection of HIV specific antibodies. They had relatively high sensitivity but poorer specificity than was required for efficient screening program.

The second generation assays used HIV recombinant antigens (HIV capsidic and envelope proteins) as the target antigen bound to the solid phase and also incorporated a recombinant antigen for HIV-2. These assays had a high sensitivity and improved specificity with respect to the first-generation EIAs.

First-and second-generation tests have a window period of about 6 to 12 weeks for most individuals (Fig.2).

Third generation assays relies on a sandwich technology, where IgG or IgM antibodies from the specimen bind both to recombinant peptides adsorbed on the solid phase and to enzyme-conjugated recombinant antigens. The ability of these

assays to detect IgM, in addition to IgG, results in a estimated reduction of the antibody-negative window period to 20-30 days. In addition, third generation tests have the capacity to detect certain HIV subtypes, particularly HIV-1 Group O which were not included to previous generation tests [66].

Fourth generation assays combine IgM and IgG antibody and viral capsidic p24 antigen detection, further reducing the timing of identification of HIV infection to two weeks and making detection of acute HIV infection feasible.

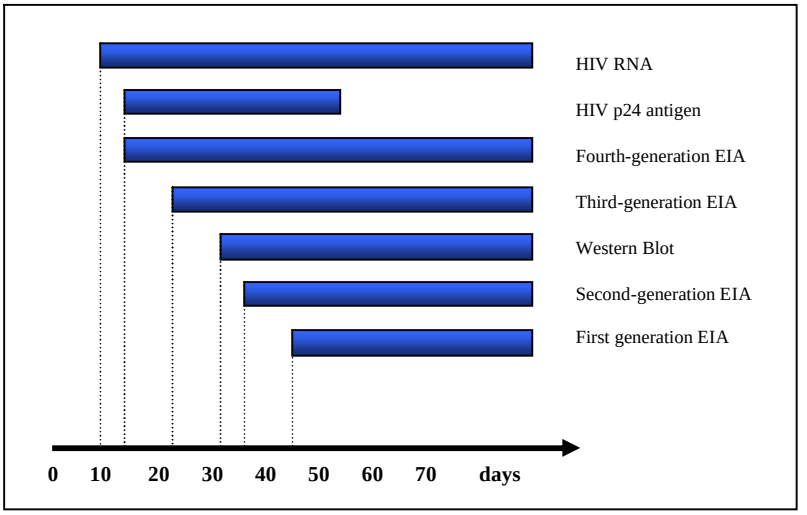


Fig. 2: Time-line of HIV infection.

They are extremely good screening test with a sensitivity of >99.5% and provide for the simultaneous detection of antibodies to both HIV-1 and HIV-2.

False positive reactions by using EIA testing may result from non-specific cross-reacting antibodies in patients with autoimmune diseases, hepatic disease, pregnancy, undergoing multiple transfusions or recent immunization, particularly for the first generation assays.

Rapid serologic HIV tests are defined as tests that are easy to perform, do not require instrumentation, provide results in less than 30 minutes and can be read visually, proving to be as accurate as the conventional EIAs [78,79].

Evaluations of these tests throughout the 1990s up to the present indicate generally good performance regardless of the format: currently available rapid HIV tests have demonstrated sensitivities and specificities comparable to those of standard HIV testing (more than 99%). Rapid tests are important tools for surveillance, screening, and diagnosis, and can be reliably done on plasma, serum, whole blood, or saliva [80].

As with EIA, the higher the prevalence of HIV within a population, the higher the positive predictive value of the rapid test. However, the positive predictive value of a combination of HIV rapid tests is nearly 100% in regions of low HIV prevalence. Thus, when used in combination, rapid tests can increase both the speed at which results are reported, while minimizing the number of false positive. Moreover, the negative predictive value of a single rapid test in regions of low HIV prevalence is high.

Rapid HIV tests have proven particularly useful in certain situations, when results are needed with fast turnaround to allow prompt provision of antiretroviral treatment to prevent HIV transmission, including labour and delivery settings with women of unknown HIV serostatus and employee exposures [81,82].

Rapid testing offers the convenience of point-of-care testing, providing patients with test results and immediate counselling during the initial visit. These rapid HIV tests are increasingly being used in various clinical scenarios to decrease the

number of missed opportunities for detection of HIV-infection. Their use is particularly applicable in specific clinical and non-clinical settings; public health settings; labour and delivery wards; in the management of occupational exposures; and in resource-constrained settings.

The two limitations of these serological tests are detection of infection during primary infection when antibodies are absent, and in infant younger than 18 months who might bear maternal HIV-1 antibodies.

The most extensively used formats suitable for rapid HIV testing are: immunoconcentration, immunochromatography and particle agglutination.

Immunoconcentration assays are flow-through devices, based on simplified second-generation EIAs technology. They use HIV-1/2 specific peptides immobilized onto a solid phase with an underlying absorbent material containing a coloured marker. Diluted specimens are placed onto the membrane surface where HIV antibodies are captured and can be visually detected, as a coloured spot or line. These tests are provided with device controls to indicate the proper performance of the test steps and reagents.

Immunochromatography tests are lateral-flow devices, that are usually based on third-generation technology [83]. They consist of a test strip that has HIV antigen immobilized onto a membrane surface, with an underlying absorbent pad impregnated with coloured reagents that permit the visual detection of anti-HIV antibodies. Specimens are placed in the tip of the strip and allowed to diffuse along the test region where HIV-specific antibodies will be captured and identified by the coloured reagent.

Particle agglutination assays rely on several different particle types (such as gelatine or latex) that are coated with HIV antigens. When mixed with a serum or plasma specimen, HIV antigen-coated beads are cross-linked into visible aggregates in the presence of HIV antibodies.

All these assays contain antigens for the detection of antibodies to HIV-1/2 and several of them assays permit the differentiation of HIV-1/2 infections by separating the two antigens on the strip.

4.1.2 *Confirmation testing for HIV*

The Western Blotting (WB) procedure is the most extensively used confirmation assay. It is a highly specific immunoblot that allows for the detection of antibodies to specific capsidic and envelope HIV proteins. It relies on an electrophoretic technique to separate HIV antigens derived from lymphocyte-cultured viral lysates onto nitrocellulose sheets by molecular weight.

HIV antibody-positive strips produce a characteristic pattern of bands related to antibodies direct against specific HIV proteins [80].

A positive Western blot result is defined by the presence of any two of the following bands: p24, gp41 or gp120/160 for HIV-1 and gp34 or gp105 for HIV-2. The assay is interpreted as negative by the absence of any reactivity [69,71,84].

As established by the interpretative criteria set by the CDC, a reactivity profile that does not meet criteria for either positive or negative results is considered indeterminate [85].

A reactive EIA testing, together with a positive Western Blot display a positive predictive value of greater than 99.99%.

Line Immunoassay (LIA) is a confirmatory assay that incorporates separate recombinant HIV antigens on nitrocellulose strips so that each positive reaction result due to the presence of specific HIV antibodies can be visualized separately.

The LIA offers the advantage of eliminating the background from non-specific host cell proteins, as possible when using viral lysates, thereby decreasing the number of indeterminate results in non-infected persons.

Indirect Immunofluorescence Assay (IFA) allows for anti-HIV antibody detection, by using HIV infected cells (usually lymphocytes) fixed to a microscope slide. Serum is added and, if present, the anti-HIV antibodies react with the intracellular HIV before being detected with the fluorescent conjugate (anti-human immunoglobulin coupled with a fluorescent tag). This technique has the advantage of sometimes providing definitive results of samples that have yielded indeterminate results by Western blot analysis, but has the disadvantage of requiring an expensive and well-maintained fluorescence microscope and of a subjective interpretation.

4.1.3 *Direct detection of HIV virus*

During acute HIV infection, prior to the appearance of antibody, HIV infection can be diagnosed by the demonstration of circulating p24 antigen, or by the presence of viral RNA or DNA.

At present, the use of p24 antigen capture assay is very limited for diagnostic purposes, having been replaced with more sensitive nucleic acid assays [76].

Nucleic acid testing (NAT) is increasingly being used as a sensitive method to detect neonatal infections and also to identify acute antibody-negative/RNA

positive infections in high risk symptomatic adults [86,87]. The major NAT methods include the reverse transcription Polymerase Chain Reaction (rt-PCR), Nucleic Acid Sequence Based Amplification (NASBA) and signal amplification branched-chain DNA (bDNA).

Although antibody tests constitute the mainstay of HIV diagnosis, antibody testing alone may fail to identify highly-infectious individuals during the first weeks after HIV infection. Viral nucleic acid detection can play a valuable role in identifying early infection within 9-11 days following exposure, before seroconversion occurs, thus allowing for early treatment and prevention of secondary transmission [88].

However, HIV nucleic acid testing hasn't a 100% sensitivity and specificity: false positive results may occur in 2-5% of HIV NATs, especially when low RNA levels are detected, and false negative results may be reported in individuals with very recent HIV acquisition, depending on the existence of a narrow window period. In these cases, risk-reduction behaviour associated with repeated testing should be recommended [89, 90].

In addition to screening for HIV, molecular techniques provide quantification of viral nucleic acid in plasma (viral load), that is a strong predictor of the disease progression rate, thus establishing initial prognosis, determining the need for therapy and monitoring the effectiveness of anti-retroviral treatment [76]. These tests are very sensitive and the most used assays have a detection limit of 50 copies/mL.

4.2 HIV testing in surveillance

Serosurveillance data are used to estimate HIV prevalence rates and geographical distribution of infection, as well as to monitor trends over time in specific population groups at increased risk for infection [91].

Linked testing confidential or anonymous is the preferred HIV testing approach, when the specimens are collected in population not accessible through clinic settings, such as injecting drug users, female sex workers and MSM.

The state of epidemic (generalized, concentrated or low-level) determines which population groups are surveyed: countries with low-level epidemics focus primarily on specific population groups who are perceived to be at high risk for infection and venues attended by target groups are selected as sentinel sites (drug treatment centres, bars, discos), whereas sentinel serosurveillance among pregnant women at antenatal clinics are conducted in countries with generalized epidemics.

Current UNAIDS/WHO recommendations on selection and evaluation of appropriate HIV testing technologies for surveillance purposes include a serial two-test strategy irrespective of HIV prevalence in the population being studied [91-94] (Fig.3).

For those surveillance settings where test results are provided to survey participants, samples are initially tested with a high sensitive screening assay and reactive samples are retested with a second test.

The first test should have as high a sensitivity as possible (at least 99%) and the second test should have as high a specificity as possible (at least 98%).

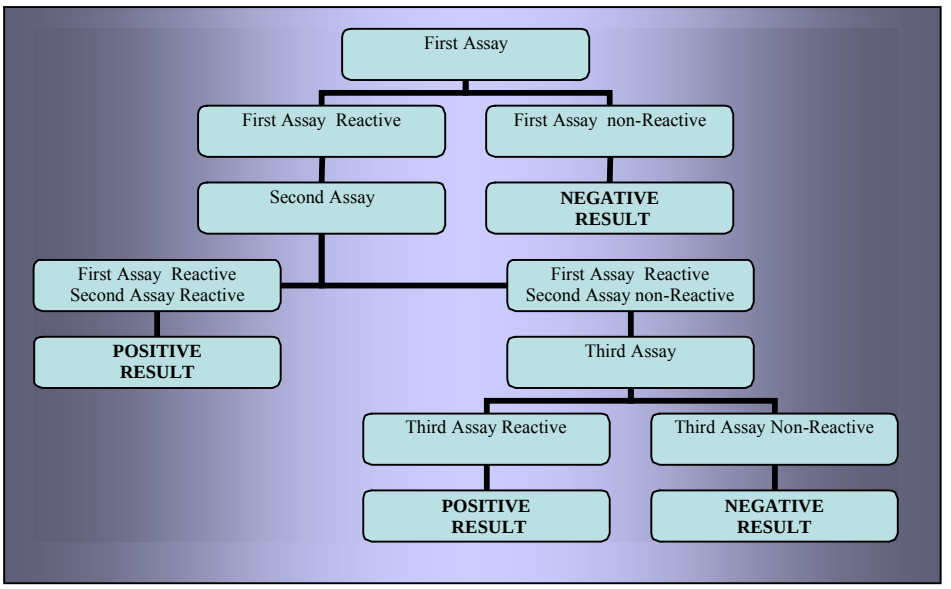


Fig. 3: Algorithm for HIV diagnoses in surveillance.

The first test is the screening test, so it is designed to detect all positives. A second test is used to confirm whether specimens found reactive with a particular screening test contain specific HIV antibodies. Because a few false positive results may occur, the second test or confirmation test needs to be highly specific to ensure that all truly negative test results are identified as negative [94,95].

For surveillance, current testing procedure use latest generation enzyme immuno-assay (EIA) tests, rapid tests based on different antigen preparations, or a combination of both. The preference of format will depend on several factors: EIA testing is usually preferred and cost-effective when larger numbers of specimens are to be run and when they can be batch processed; on the contrary, rapid tests may be preferred when small numbers of samples are being tested and a short turn-around time to provide results are available ("on site" testing).

A third different test ("tie-breaker") should be carried out as a definitive confirmation assay in the event of discordance between the first and the second tests. Western blot can be suitable for this purpose. If Western Blot is negative, the result of the screening test is considered to be a false positive, indicating that the patient is not infected with HIV. If the confirmatory test is positive, a report is sent back with a confirmed positive statement. Indeterminate results may be found up to 10% to 20% of cases and may be due to recently acquired HIV infections or to the interfering action of cross-reactive antibodies. Re-testing guidelines for indeterminate results recommend HIV testing to be repeated from 2 weeks to two months later for a definitive result.

Summing up, current guidelines recommend two different tests with a third test as a tie-breaker if the first two are discordant. Since a second sample for confirmation may not be feasible in surveillance settings being considered for hard-to-reach population, HIV determination is carried out by multiple tests on a single sample.

Samples are considered positive if they are reactive on two different tests. Samples that are not reactive with the first test are considered negative, as are those reactive on test one but non-reactive on both tests two and three.

4.3 Oral fluid testing

Although the serological identification of antibodies to HIV in blood is considered the most extensively used method to detect HIV infection, in the context of epidemiological surveys, it is impractical to collect samples of venous blood for HIV testing [96,97]. Specimens should be obtained with minimal training and

minimal risk of infection and, sometimes under difficult field conditions. In addition, blood sampling may reduce participation and venous puncture may be difficult in certain groups, such as injecting drug users or subjects with a poor venous access [98].

Recently, however, the oral fluid testing been advocated as a non-invasive alternative to blood testing for the detection of antibodies to a number of specific infectious diseases, including HIV infection [99-105].

For HIV prevalence surveys, where compliance with venipuncture may be low, the safety, the easy and non-invasive nature of collection, thereby obtaining large numbers of specimens within a short time period along with relatively low cost, make oral fluid-based methods an attractive alternative strategy to venipuncture and serological testing [106-109].

As a safe, non invasive and painless method, oral fluid testing collection offers a potential for a higher degree of acceptability among subjects being tested for surveillance purposes compared to drawing blood [100].

Therefore a greater percentage of the target population may agree to be tested, thus improving access to HIV screening and reducing the risk of selection bias [92].

Young children represent an important group where oral fluid samples are currently used as a non invasive alternative to serum for the surveillance of certain virus infections, particularly those that are vaccine preventable. In the UK, oral fluid sampling has been used for the surveillance of measles, mumps and rubella (MMR) since 1994 [110].

However the main disadvantage when using oral fluids as testing samples is that the concentration of specific antibodies may not be sufficient to detect truly positive cases [92].

Oral fluid is a complex body fluid consisting of secretions from salivary glands and transudate from mucosal surfaces and the gingival crevice – so called gingival crevicular fluid– [100, 111,112]. Gingival crevicular fluid is a serum transudate that is derived from the continuous seepage of fluid from the gingival capillaries into the gingival crevice between the gum margin and the teeth. This fluid is rich in plasma-derived IgG, the predominant class of anti-HIV antibodies. Since the first prerequisite to an effective oral HIV test is to isolate a sample rich in IgG, hence an oral fluid sample that is well represented with crevicular fluid is most suitable for antibody testing [99,113]. The levels of IgG present in GCF vastly exceed those found in whole saliva (300:1) and are about 4 times less than those in serum (3,500 mg/L vs. 12,500 mg/L) [100,111-114]. Thus, GCF appears to be the main source of salivary IgG and the gingival crevice is an identifiable anatomic site that provide assayable source of IgG.

However, the final IgG concentration found in oral fluid is about 1/800-1/1000 that of serum, because of the dilution effect of pure saliva, necessitating extremely sensitive tests that are able to detect small quantities of antibody. This means that sensitive assays in combination with absorptive collectors designed to be placed in specific areas of the mouth are needed to detect anti-HIV antibodies in oral fluid [100,115]. Specialized devices have been developed to standardize the collection method of oral fluid specimens in detecting antibodies to HIV-1/2.

They specifically collect oral fluid from areas of the mouth rich in crevicular fluid, by creating an osmotic gradient that enhances transudation of IgG across the mucosal surface [116].

Oral fluid collection devices involves placing either an absorptive pad along the tooth-gum margin for two minutes (i.e. OraSure; Orasure Technologies, Bethlehem, PA, USA) or a cotton roll under the tongue until an adequate amount of whole saliva is collected and an indicator of device turns blue (i.e. Omni-SAL; Saliva Diagnostic Systems, Vancouver, WA) or to suck a sponge (i.e. Salivette; Sarstedt Ldt., Leicester, UK) or a polystyrene sponge to be rubbed along the base of the gums, using an action similar to tooth-brushing (Oracol, Malvern Technologies, Worcester, UK) [110,117]. The first two devices include a preservative solution to maintain specimen stability.

The first studies carried out on whole saliva by using first-generation EIA testing or Radio-Immuno-Assay for HIV detection in seropositive patients yielded sensitivities of only 57-70% [99,118].

Modification of existing assays by increasing sample volume, decreasing the diluent volume or by lowering optical density cut-off to accommodate the use of saliva has resulted in sensitivities (97.9-100%) and specificities (100%) comparable to those observed in serum testing [119,120].

The first assays developed specifically for testing oral fluid were IgG capture radio-immunoassay (GACRIA) and IgG antibody capture ELISA (GACELISA), based on the principle of concentrating specific IgG from saliva by capturing them on a solid phase, so that even small amounts of HIV-specific IgG may be

revealed. Using optimal dilutions, GACELISA has shown sensitivities of 98-100% and specificities around 100% [99,121-124].

Similarly, GACRIA exhibited sensitivities of 94.4-100% and specificities of 98.8-100%, depending on the method used, when whole saliva was used [122,125].

The introduction of specifically designed oral fluid collection devices that concentrate and stabilize the salivary-associated immunoglobulins, together with appropriate testing assay resulted in sensitivity of 97.8-100% and specificities of 99.2-100% [116,126-133].

Rapid and simple "point-of-care" tests have also been used successfully to test oral fluids for HIV. Oral fluid-based rapid testing is a very important component of HIV control initiatives and programs [134].

In particular, Oraquick® Advance Rapid HIV-1/2 antibody test (OraSure Technologies), a qualitative, visually read lateral-flow immunoassay, was the first rapid HIV test approved by FDA for the detection of HIV-1/2 antibodies in oral fluid as a point of care (POC) test [135]. The test is reported as having 99.3% sensitivity and 99.8% specificity [136]. Post-marketing surveillance of the OraQuick Advance Rapid HIV-1/2 Antibody Test on whole blood and oral fluid yielded favourable results. Testing over 135,000 whole-blood samples and over 26,000 oral fluid test yielded a specificity of 99,98% with a positive predictive value of 99,24% for blood, and specificity of 98,89% with a positive predictive value of 90% for oral fluids [137].

However, oral fluid rapid testing have some limitations: i. they cannot be used to perform additional testing for confirmation or for special studies; ii. they need

trained medical staff at the point of care for interpretation of test results; iii. they have a higher cost per test than EIAs.

OBJECTIVE

In epidemiological surveillance, oral fluid testing appears more appealing than serological testing carried out on blood samples, due to its good feasibility in terms of ease of sampling collection, safety and acceptability to hard-to-reach-population, as well as its potential for a performance in antibody assays comparable to those obtained with serum testing. In particular, EIA testing may be a cost-effective tool for HIV prevalence estimation in epidemiological studies, where large number of samples are processed at a time, with the additional possibility for confirmation testing on the same sample.

This study was designed:

- To estimate the prevalence of HIV infection and associated socio-demographic and behavioural factors in a cohort of MSM enrolled in seven European countries by means of non-invasive oral fluid sample collection. Understanding the factors that may contribute to the early identification of HIV infections will allow for implementation of evidence-based preventive interventions.
- To proceed to validation of a third-generation EIA test, CE marked for use with serum, (EIA GENSCREEN HIV 1/2 version 2;BIORAD) for detecting antibodies to HIV-1/2 on oral fluid samples. Performance evaluation was carried out by testing paired oral fluid and serum samples obtained from confirmed HIV positive patients and controls. This procedure was instrumental to ensure that the laboratory technique was able to reproduce,

when applied to oral fluid specimens, values on sensitivity and specificity, such as those obtained when serum was used.

MATERIAL AND METHODS

The study was undertaken in two phases: the validation study, followed by a multi-centre survey.

It involved the participation of health institutions from seven European countries:

- Czech Republic : National Institute of Public Health, Prague
- Greece : Hellenic Centre for Infectious Diseases, Athens
- Italy : Regional Centre for Health Promotion, ULSS 20 Verona; Department of Pathology, Section of Immunology and Microbiology, University of Verona; AIDS/STI Centre, ASL 2 Isontina, Gorizia
- Romania : Public Health Institute, Timisoara
- Slovakia : National Reference Centre for HIV/AIDS, University of Bratislava
- Slovenia : Regional Centre for Health Promotion, Maribor; Dermatovenerologic Clinic of University of Ljubljana
- Spain : AIDS/STI Epidemiological Centre, Catalonia, Barcelona

1. VALIDATION STUDY

1.1 Study settings and participants

The study population included 498 subjects, of which 263 were HIV positive patients attending health facilities (hospital, labs) for treatment and/or follow-up and 235 were confirmed HIV negative individuals recruited among volunteers.

Participants were eligible if any of the following criteria were met: i. adult age (≥ 18 years old); ii. previously known HIV positive patients attending health facilities regardless of being in HAART therapeutic regimen or confirmed HIV

negative subjects, if recruited as negative controls; iii. willingness to sign an informed consent form to take part in the study; iv. accepting to provide both an oral fluid and blood sample.

Demographic, behavioural and clinical data were collected by means of a structured and anonymous questionnaire administered by health and lab professionals in each participating country.

Patient anonymity was guaranteed, by labelling the samples and the information form with a computer-generated identifier barcode.

1.2 Sample collection and testing

The oral fluid samples were collected by using the Oracol device (Malvern Medical Development, Worcester, UK), which consists of an absorbent foam swab fixed to a plastic stick and conserved in a capped tube. The swab is specifically designed to concentrate the GCF from whole saliva, targeting the gums (Fig. 4). Patients were asked to rub the sponge firmly along the gum at the base of the teeth for approximately 1 minute, like a toothbrush.

Fig. 4: Oracol collection device.

The serum samples were obtained at the same time by venepuncture.

After collection, samples were kept frozen at -20°C until shipment to the Verona University Hospital Laboratory. A tag with computer-generated identifier barcode was attached to the biological sample tubes and to the information form.

1.2.1 *Processing of oral fluid samples*

On receipt to the laboratory, tubes containing the oral fluid swab were filled with 0.5 ml of transport medium (phosphate-buffered saline, pH 7.2 with 10% foetal calf serum, 0.2% Tween 20, 0.5% gentamicin and 0.2% fungizone) and let stand at 4°C for 1 hour. Swabs were then removed from tubes, squeezing and twisting the sponge against the plastic wall to drain as much liquid as possible. Oral fluid was recovered by two turns of centrifugation at 2500 rpm for 10 minutes and at 14000 rpm for 1 minute, collected in sterile test microtubes and then stored at -20°C, until testing.

1.2.2 *Enzyme Immuno-assay (EIA) for detection of anti-HIV 1/2 IgG*

All serum and oral fluid paired samples were tested for antibodies to HIV-1/2 by using EIA GENSCREEN HIV 1/2 version 2 (BIORAD).

It is an enzyme immuno-assay based on a sandwich technology, intended to detect anti-HIV1 and/or anti-HIV2 antibodies in human serum or plasma. It is based on the use of a solid phase, coated with purified recombinant antigens (HIV1 gp160 and gp25 proteins and a peptide corresponding to an immuno-dominant epitope of HIV2 envelope glycoprotein) and a recombinant antigen-peroxydase compound (peptides corresponding to HIV1/2 immuno-dominant epitopes of the envelope glycoproteins and nucleocapsidic proteins).

Briefly, 75µL of undiluted oral fluid and serum samples were added to 25 µL of specimen diluent in microtiters, along with positive, negative and cut-off serum controls. The microtiter plate was then incubated in a microplate incubator at 37°C for 30 ± 5 minutes to allow for binding of anti-HIV 1/2 potentially present in the sample to the antigens fixed to the solid phase. The plate was then washed and incubated for 30± 5 minutes with 100 µL of peroxydase-conjugated purified HIV1 and HIV2 antigens at room temperature. After washing, 80 µL of a substratum revealing the presence of the enzyme-conjugated antigens was added and incubated for 30± 5 minutes at room temperature in the dark. The reaction was stopped by adding 100 µL of a sulphuric acid solution. Finally, the resulting absorbance values (OD) were read with a spectrophotometer at 620-700 nm of wave of length.

On oral fluid samples, no dilutions were made and oral fluids were added to wells corresponding to the total volume of serum samples.

The manufacture's controls were used to determine the cut-off for serum samples. Cut-off values for oral fluid specimens were given by calculating the mean±SD absorption values (OD) of all oral fluid controls.

A sample was identified as positive if the ratio sample optical density to cut-off was greater than 1.

1.2.3 Testing of oral fluid sample quality by determination of total IgG

To assure that the oral fluid sample found negative after testing was taken properly and in sufficient quantity, a total IgG quantification test was carried out.

The minimum IgG concentration that an oral fluid sample had to contain to be acceptable for the oral fluid assays was 3.5 mg/L [110,112,113].

The total IgG content of oral fluid samples was determined by using an enzyme immuno-assay (Human IgG ELISA Quantification kit; Bethyl Lab. inc).

The assay procedure involved the following steps:

- Microplate loading with 100 µL of oral fluid samples diluted 1:250 and standard dilutions of human IgG to be assayed in duplicate;
- Coating microtiter well strips with capture anti-human IgG antibody (purified goat anti-human IgG antibody);
- Microplate incubation at room temperature for 1 hours and washing out of unbound sample material;
- Addition of horseradish peroxydase (HRP) labelled anti-human IgG conjugate;
- Microplate incubation at room temperature and washing out with wash solution;
- Incubation with a tetramethylbenzidine (TMB) substrate revealing the presence of the bound conjugate by a blue reaction product.
- Addition of sulphuric acid to stop the reaction;
- Detection of the reaction by reading the absorbance values with a microtiter plate reader at 450 nm of wave of length. Individual specimen IgG concentration were extrapolated from the averages of absorbance readings of each set of oral fluid samples tests on a standard curve, created by using Softmax Pro 4.325 (Life Sciences Edition).

1.3 Statistical analysis

The main outcomes were diagnostic accuracy measures of EIA testing on oral fluids, estimated by using sensitivity, specificity and predictive values, along with 95% confidence intervals (CI). The detection of anti-HIV antibodies in serum samples with Genscreen was used as gold standard.

Concordance between results obtained with oral fluid and matched serum samples was established by using the kappa statistics. The following interpretation of the kappa value was used: k<20% poor, k=20-40% fair, k=41-60% moderate, k=61-80% good, k=81-100% excellent agreement. The diagnostic testing accuracy was obtained by determining the ROC area.

Cut-off values were calculated as the mean of the OD controls +3 and +5 standard deviations (SD). A t-test was performed for comparison of oral fluid and serum mean OD/CO ratios. Analysis was carried out by using Microsoft Excel 2003.

2. MULTI-CENTRE SURVEY

2.1 Study settings and participants

A multi-centre cross-sectional survey for HIV prevalence determination by using oral fluid was undertaken among 2688 MSM between April 2008 and September 2009. Recruitment and sample collection occurred in preliminarily mapped venues (bars, discos, clubs) in each country.

Participation was voluntary and anonymous. Respondents were eligible if any of the following criteria were met: i. adult age (≥ 18 years old); ii. accepting to provide an oral fluid sample and to fill in a questionnaire.

They were informed of the aims of the study and were given a card with information on the closest HIV screening and counselling centre, where people can get the test result and receive post-test counselling. Since the test result based on oral fluid sample is not meant to be diagnostic, volunteers were informed that they should be tested again with another test performed on serum or oral fluid according to their wish. A structured questionnaire to obtain information concerning demographic and behavioural data was administered by trained personnel.

Barcode tags were stuck on biological samples and on the corresponding questionnaire to enable reliable tracking without using personal data to identify subjects.

2.2 Sample collection and testing

Oral fluid specimens were obtained with the Oracol device (Malvern Medical Development, Worcester, UK) and tested for antibodies to HIV-1/2.

Sample storage and processing was performed as described above in the validation study.

2.2.1 Enzyme Immuno-assay (EIA) for detection of anti-HIV 1/2 IgG

Oral fluid samples were tested for anti-HIV 1/2 antibodies by using EIA GENSCREEN HIV 1/2 version 2 (BIORAD).

2.2.2 Total IgG quantification

To ensure that the oral fluid sample found negative after testing was taken properly, a total IgG quantification test was carried out. An oral fluid sample had

to contain at least 3.5 mg/L IgG to be acceptable for the oral fluid assays, otherwise it was excluded from data analysis.

2.3 Statistical analysis

HIV prevalence was defined as the number of confirmed HIV seropositive subjects divided by the total number of HIV-tested individuals during the study period. We also calculated 95% interval confidences (95% IC).

A chi-square test was carried out for comparison of prevalence rates.

Socio-demographic (age, educational attainment, environmental context) and sexual behavioural data (self-reported serostatus, STI history) were evaluated in association with HIV-1/2 positivity.

Statistical analysis was performed by using Microsoft Excel 2003 package.

RESULTS

1. ALGORITHM FOR VALIDATION OF HIV ORAL FLUID TESTING

Oral fluid samples, that tested positive by EIA Genscreen were compared to serum EIA results. If serum specimen was reactive for HIV-1/2 antibodies, a positive concordant result was reported (Fig.5). On the contrary, if serum specimen displayed non-reactivity to EIA testing and belong to the control group, the oral fluid testing result was reported as false-positive for HIV.

In the event of oral fluid sample non reactivity to EIA testing, IgG content was determined to assure that the negative result was reliable. Only valid samples were compared to serum results. If serum assay was concordant with oral fluid testing, a negative result was reported. On the other hand, if serum testing was reactive, the oral fluid assay was considered a false negative result.

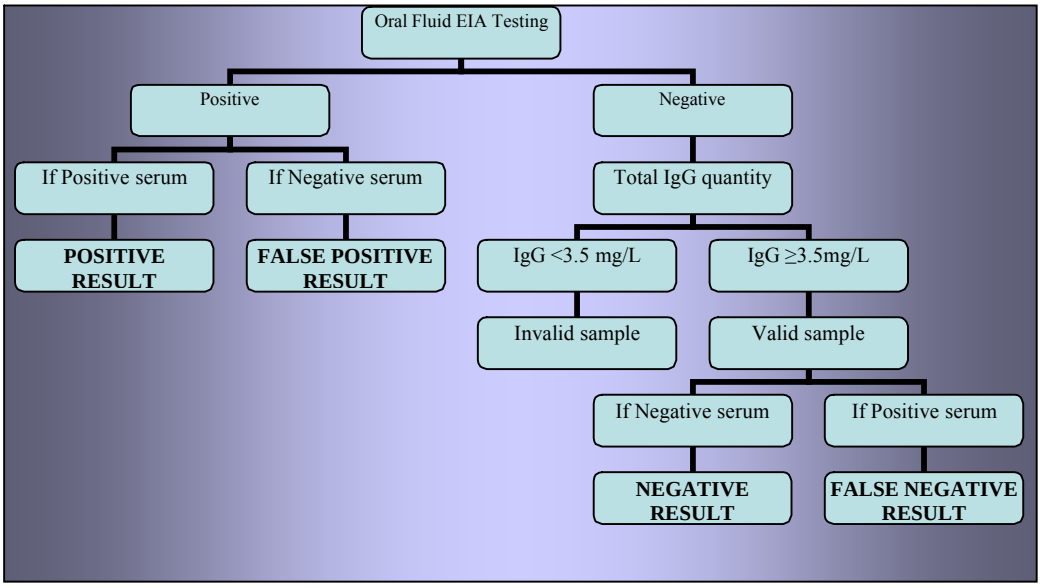


Fig. 5: Validation of HIV oral fluid testing.

2. SURVEY HIV TESTING STRATEGY

Oral fluid samples yielding a positive result to EIA were tested for confirmation by using an immunochromatographic assay (Determine HIV-1/2; Inverness Medical, Abbott Laboratories; Abbott Park, IL) (Fig. 6).

The immunochromatographic assay is a visually read, qualitative assay that is based on the use of recombinant antigens and synthetic peptides specific for HIV-1/2 envelope proteins. If antibodies to HIV-1/2 are present in oral fluid sample, they bind to an antigen-selenium colloid conjugate and to HIV-specific antigens at the test location, causing a red line to appear.

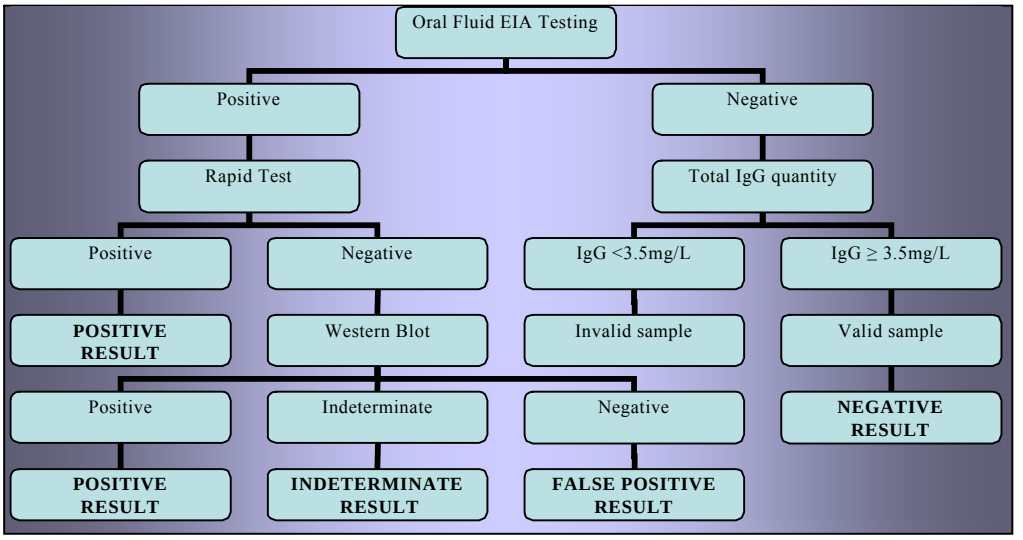


Fig. 6: Algorithm for oral fluid testing in surveillance.

If both EIA and rapid test were reactive, the result was reported as positive for HIV infection. If the rapid test was non-reactive, oral fluid specimens were subjected to Western blot analysis (HIV Blot 2.2; MP Biomedicals Asia Pacific Pte Ltd., Singapore) for definitive characterization. The WB combined purified

HIV-1 antigens (corresponding to the structural products of gag, pol, env genes) with an HIV-2 synthetic peptide in one strip. CDC criteria for determining western blot results were used to assign the final status of such samples [83].

On the other hand, oral fluid samples showing no reactivity to EIA testing were checked for total IgG concentration. If IgG content was sufficient, a negative result for HIV-1/2 antibodies was reported.

3. VALIDATION STUDY

3.1 Soundness of commercial kits used in the validation and survey analytical sessions

3.1.1 Inter-assay reproducibility of Genscreen HIV 1/2 version 2 (BIO-RAD)

No significant OD variability in positive (mean: 3.06±0.27) and negative (mean: 0.16±0.03) control sera was observed among different analytical sessions.

The inter-test variability of positive and negative control sera provided by the kit manufacturer is shown in Figure 7.

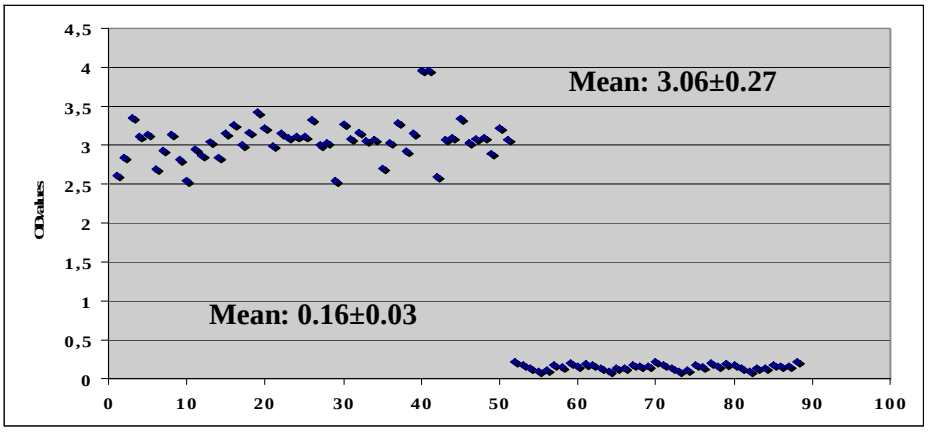


Fig. 7: Distribution of EIA Genscreen HIV-1/2 control sera.

3.1.2 Inter-assay reproducibility of Human IgG quantification kit

The standard curve points corresponding to human IgG standard dilutions (500-7.8 ng/ml) were generated with good reproducibility among independent runs. The inter-test variability of reference curve provided by the kit manufacturer is described in Figure 8.

ng/ml	OD values
500	2.47±0.007
250	2.2±0.09
125	1.8±0.08
62.5	1.1±0.06
31.25	0.61±0.04
15.625	0.27±0.02
7.8	0.14±0.04

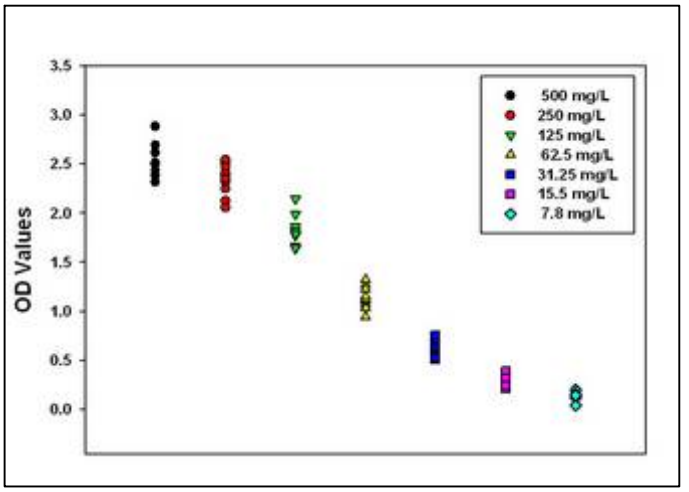


Fig. 8: Standard curve of Human IgG quantification EIA.

3.3 Performance of oral fluid EIA testing

The sample set included 263 paired serum and oral fluid samples from confirmed HIV positive patients and 235 samples from HIV negative subjects.

The contribution from each European country is shown in Table IV.

Table IV : Study population recruited by country.

Country	HIV-positive patients	HIV-negative subjects
CZECH REPUBLIC	37	30
GREECE	40	33
ITALY	38	32
ROMANIA	37	35
SLOVAKIA	37	35
SLOVENIA	37	35
SPAIN	37	35
TOTAL (N=498)	263	235

The socio-demographic profiles of individuals recruited in the study are shown in Table V and VI.

Table V: Demographic and clinical features of the HIV positive patients (N=263).

Age (mean ±s.d.; range)	**39.9±10.9 ys (20-75 ys)**
Gender	**56 females, 205 males**
HIV risk factors	**Heterosexual : 68/263 (26%)** **MSM : 139/263 (53%)** **IDU : 19/263 (7%)** **Unknown : 37/263 (14%)**
Median time from HIV seroconversion (years)	**7,8 ys**
Antiretroviral treatment (HAART) (%)	**164/263 (62.4%)**
Previous STI (%)	**62/263 (23.6%)**

Table VI: Demographic data of the HIV negative subjects (N=235).

Age (mean ±s.d.; range)	**33.6±11.2 ys (19-86 ys)**
Gender	**83 females, 150 males**

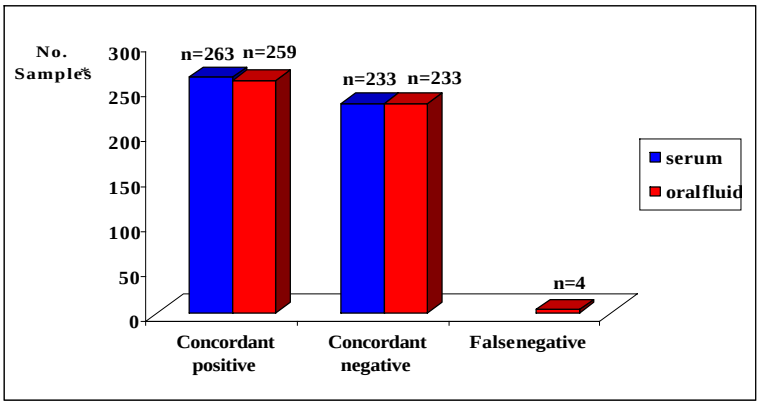
The mean age of the HIV-positive patients was 39.9±10.9 years (range:20-75 ys); 78.5% was male. More than half patients identified themselves as MSM; a heterosexual mode of transmission was described in a quarter of cases of HIV infection, while IDU accounted for 7% of all recruited patients. Data about HIV probable route of transmission were unknown in 14% cases. More than a fifth of patients reported a history of previous sexually transmitted infections (STIs).

Antiretroviral therapeutic regimen had been administered to more than 60% of patients enrolled.

Among the control subjects, the mean age was 33.6±11.2 years and 64.4% were male.

HIV antibodies were determined by using EIA Genscreen HIV 1/2 and the oral fluid samples were tested in the same way as the sera, following the manufacturer's package instructions.

Among the HIV seropositive patients, antibodies to HIV were detected in 259 out of 263 (98.5%) oral fluid samples and in all 263 (100%) matched sera by Genscreen (Fig.9,10).



(*): number of samples considered appropriate for testing

Fig. 9: Oral fluid testing results vs. serum by using EIA Genscreen.

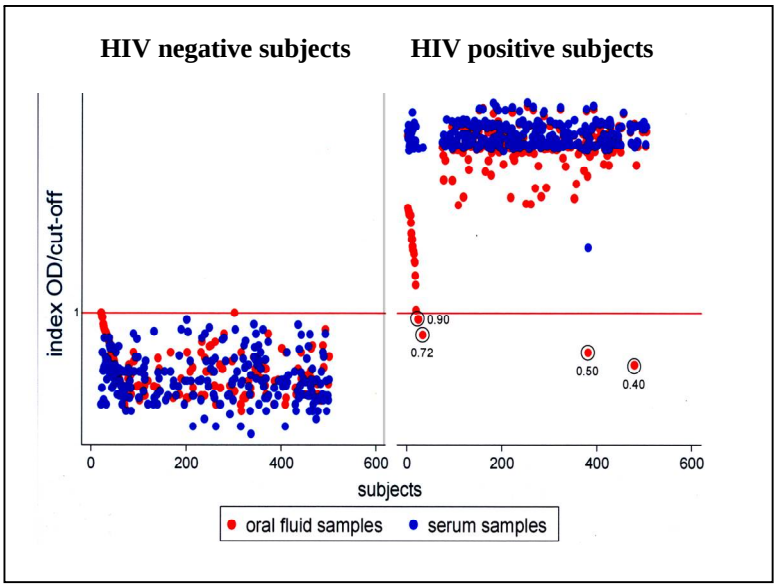


Fig. 10: Performance of EIA Genscreen HIV-1/2 on oral fluid samples vs. serum.

Among the HIV-seronegative group, of the 235 tested paired samples, the totality was Genscreen test negative. All oral fluid samples were subjected to an IgG quantification assay and 233 out of 235 (99.1%) provided a valid result (mean IgG concentration of 24.41 mg/L).

EIA GENSCREEN HIV 1/2 version 2 (BIORAD) performed on oral fluid specimens exhibited a sensitivity of 98.5% (95% CI: 96.2-99.6) and a specificity of 100% (95% CI: 98.4-100).

Thus, given a prevalence of more than 50% (as in this study), the positive predictive value (PPV) was 100% (95% CI: 96.8-100) and the negative predictive value (NPV) was 98.3% (95% CI: 95.7-99.5).

The positive and negative predictive values were calculated for different prevalences of HIV (Table VII). Taking into account a specificity of 100%, the positive predictive value was 100% regardless the HIV prevalence in the population. The negative predictive value ranged from 99.9% for a population with a HIV prevalence of 0.5% to 98.8% for a population with a HIV prevalence of 80%.

Table VII: PPV and NPV of HIV EIA oral fluid testing for different prevalences.

Prevalence	**0.5**	**1**	**5**	**10**	**15**	**50**	**80**
PPV (%)	100%	100%	100%	100%	100%	100%	100%
NPV (%)	99.9%	99.9%	99.9%	99.8%	99.8%	99.3%	98.8%

The concordance between results obtained in serum and oral fluid specimens was excellent according to the Kappa index (k=0.98; 95% CI: 0.968-1); the diagnostic accuracy as expressed by the ROC area was 0.992 (Table VIII).

Table VIII: Performance of EIA oral fluid testing.

Test	Sensitivity	Specificity	Kappa statistics	ROC area
Genscreen HIV 1/2	**98,50%**	**100%**	**0.98**	**0.992**

However, 4 discrepant results were obtained among serum and oral fluid samples from previously known HIV-seropositive patients. In all these cases, HIV antibodies were detected in serum but not in oral fluid samples by EIA, even after repeat testing. IgG quantification assay indicate that these samples were taken properly (IgG values were far above the limit value of 3.5 mg/L; mean IgG: 36.5 mg/L). Additionally, both sera and oral fluid specimens were verified with WB to confirm the presence of HIV antibodies. When tested by using WB, all serum samples remained positive for HIV, whereas 3 out 4 oral fluid samples fulfilled the positivity criteria for WB and 1 displayed an indeterminate result (Table IX, Fig. 11).

The 4 patients incorrectly identified as HIV negative by using oral fluid testing were recorded as receiving antiretroviral treatment, with $CD4^+$ counts ranging from 452 to 1039 cells/µL and a viral load less than 50 copies/mL.

Table IX: Results of HIV confirmation testing from previously known HIV positive subjects with negative oral fluid (OF) samples.

Subject No.	IgG (mg/L)	HIV EIA serum	HIV EIA OF	WB serum	WB OF
1	29.4	Pos	Neg	Pos	Ind
2	14	Pos	Neg	Pos	Pos
3	51.1	Pos	Neg	Pos	Pos
4	51.5	Pos	Neg	Pos	Pos

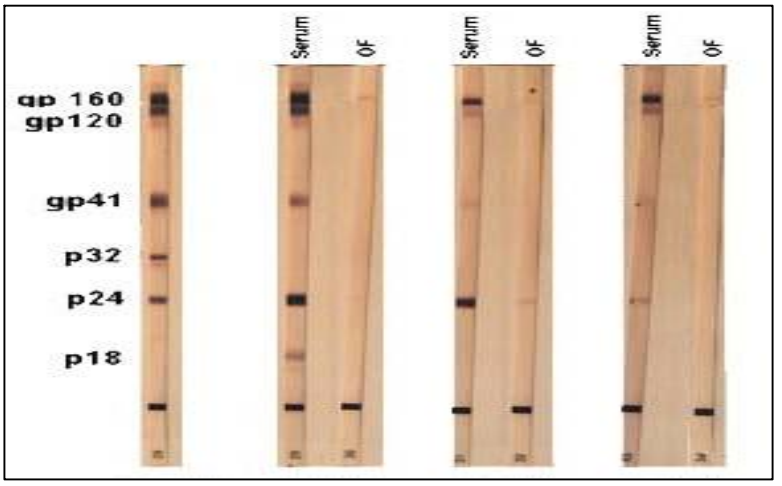


Fig. 11: Reactivity observed by WB of EIA discordant serum and oral fluid samples.

Oral fluid testing yielded a false negativity rate of 0.8%, while no false positivity rate was observed.

The mean optical densities (OD) of the 233 oral fluid controls was 0.05 and the SD was 0.03.Thus, the cut-offs for the assay using +3SD and +5SD were 0.14 and 0.20, respectively. The assay displayed the best sensitivity and specificity when a cut-off of 5 SD above the mean value for controls was used.

The distribution of optical densities in the EIA oral fluid testing from HIV-negative subjects coincided closely with that of sera from the same individuals (Fig. 12).

Our findings indicated that the cut-off value based on the control sera included in the kit was also suitable for OF testing.

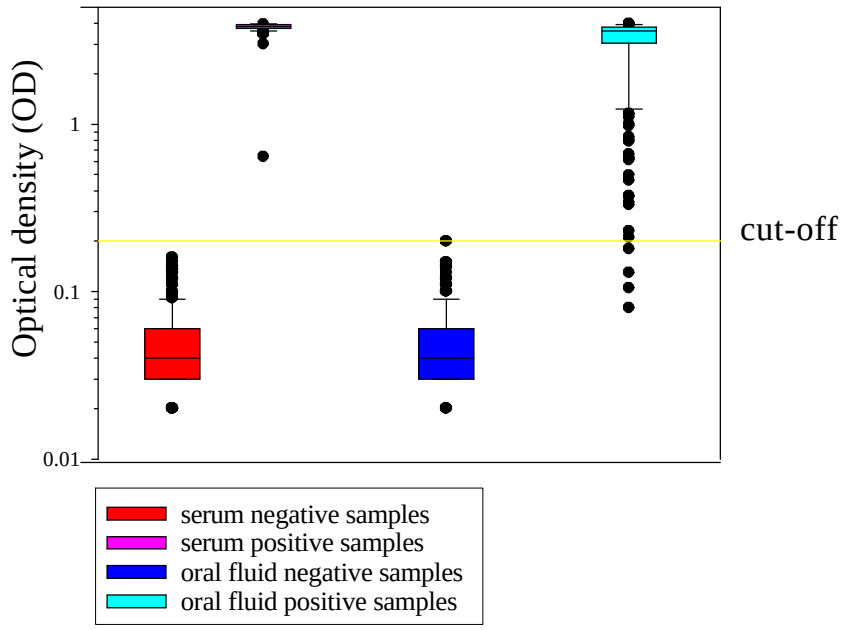


Fig. 12: Calculated cut-off value for oral fluid testing vs. serum.

The mean optical density to cut-off ratios (OD/CO) in serum and oral fluid samples among subjects testing concordantly positive, concordantly negative and falsely negative in oral fluid assay are shown in Table X.

Table X: Mean OD/CO ratio by EIA testing result.

OD/CO	Concordance negative N=233	Concordance positive N=259	Discordant N=4
Serum	0.35	24.06	14.77
Oral Fluid	0.36	20.41	0.62

The serum OD/CO of samples tested false negative by the oral fluid assay and those tested concordantly positive differed with statistical significance (14.77 vs 24.06; $p<0.001$). Similarly, the oral fluid OD/CO ratios of the false negatives were

significantly higher than those of the concordantly negative (0.62 vs 0.36; $p<0.003$).

In the attempt to improve the sensitivity of oral fluid-based assays, the minimum and maximum OD/CO values in the EIAs of the concordantly positive and concordantly negative oral fluid samples were compared to determine a lower OD/CO ratio. The maximum OD/CO ratio for concordantly negative oral fluid samples was 1; among concordantly positive oral fluid samples, the minimum OD/CO in the EIA was 1.05. Therefore, no adjustment could be done on the cut-off while testing oral fluids, without compromising the assay specificity.

These data are consistent with a high degree of concordance of Genscreen HIV-1/2 EIA testing results between serum and oral fluid specimens. No modifications of package instructions that optimized the kit for use with saliva were applied.

4. MULTI-CENTER SURVEY

4.1 Study population

A total of 2688 MSM were tested by EIA Genscreen for HIV-1/2 antibody determination on oral fluid samples and provided questionnaires (Table XI).

Table XI : Study population recruited by country.

Country	MSM enrolled in the survey
CZECH REPUBLIC	**418**
GREECE	**178**
ITALY	**500**
ROMANIA	**398**
SLOVAKIA	**396**
SLOVENIA	**399**
SPAIN	**399**
TOTAL	**2688**

The mean age of the people under investigation was 32± 9.8 years (range:18-76 ys). Respondents in Southern European countries were older than those in the Eastern countries (36±10 ys vs. 29±8 ys; $p<0.05$) (Fig. 13).

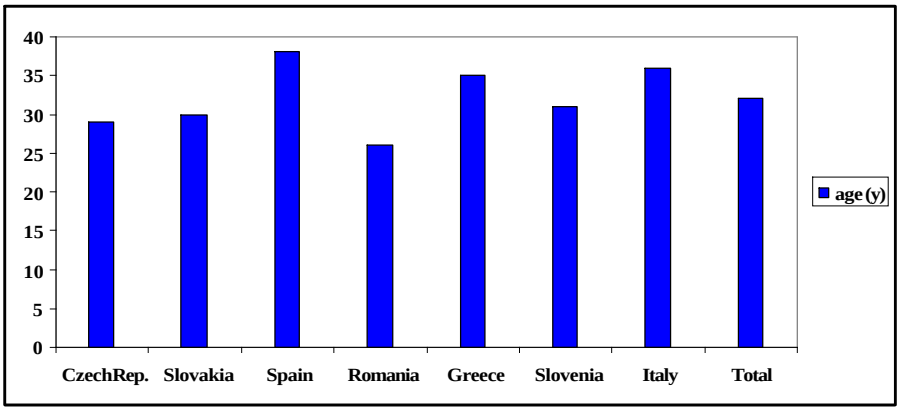


Fig.13: Age distribution of MSM enrolled in the multi-center survey by country.

More than two-thirds of participants (70%) reported living in urban settings, while the remainder were from small towns or villages. The proportion of MSM with a secondary qualification was 52%, whereas 40% had a university degree; the highest proportion of university qualifications was found in Greece (57%), while the lowest was in Czech Republic (27%).

More than 80% self identified themselves as homosexual; bisexuals accounted for 18% and heterosexuals were 2%. MSM in Italy had the highest proportion of respondents who reported being homosexual (more than 90%) and MSM in Greece had the lowest (65%) (Fig. 14).

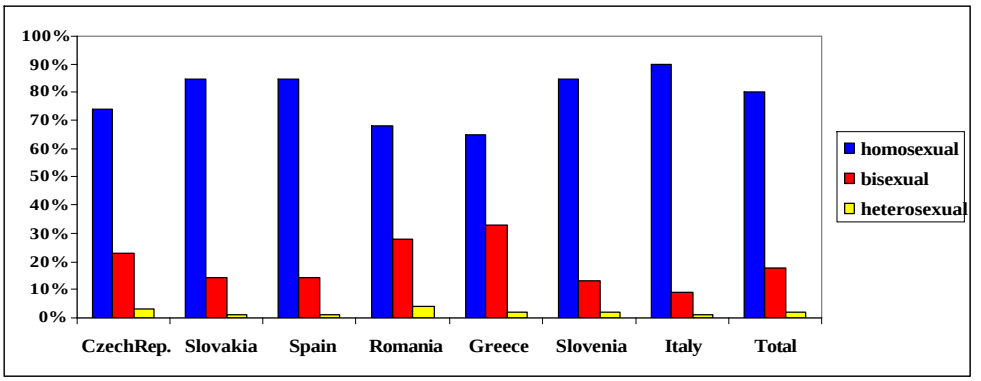


Fig. 14: Overview of respondents' sexual behaviour by country.

A history of previous STIs was documented in 4% cases for syphilis, in 5% for gonorrhoea and 4% for infections by Chlamydia.

More than a half of MSM reported HIV testing in the last 12 months; the higher proportion was found in Greece (94%) and the lowest in Slovakia (38%) (Fig.15).

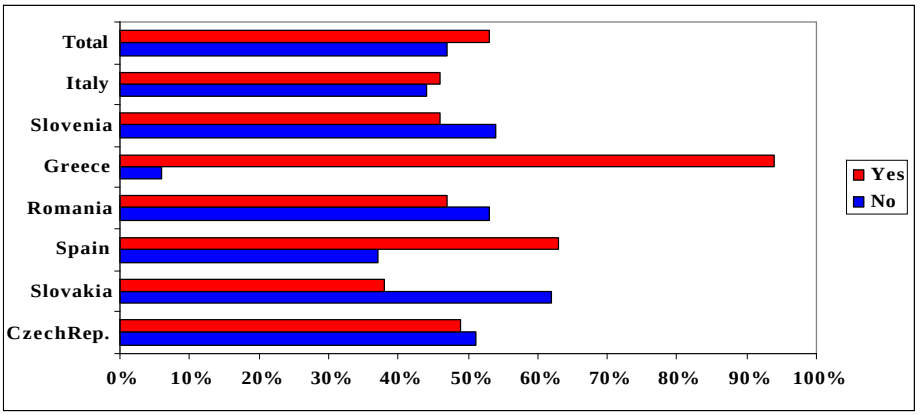


Fig.15: Proportion of subjects undergoing HIV testing in the previous 12 months.

4.2 HIV prevalence

EIA testing resulted in 208 (7.7%) reactive oral fluid samples and 2480 (92.3%) negative results (Fig. 16) (Table XII).

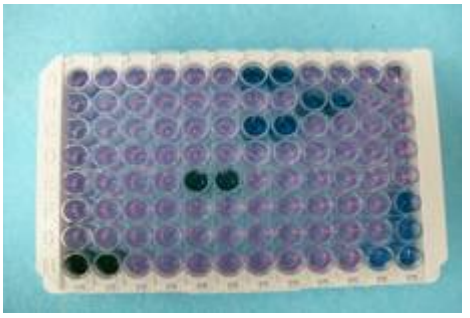


Fig. 16: EIA Genscreen HIV-1/2 microplate (blue: reactive; pink: non-reactive).

Table XII: EIA testing results on oral fluid samples.

Country		Positive EIA	Negative EIA
Czech Republic	*(n=418)*	11	407
Greece	*(n=178)*	15	163
Italy	*(n=500)*	52	448
Romania	*(n=398)*	17	381
Slovakia	*(n=396)*	23	373
Slovenia	*(n=399)*	24	375
Spain	*(n=399)*	66	333
Total	*(n=2688)*	208	2480

Of the 208 EIA reactive samples, 151 out of 208 (72.6%) were confirmed positive by a second test (rapid test). Remainder were tested for confirmation by using Western Blot (WB): 45 out of 57 oral fluid samples fulfilled the positivity criteria for WB, 2 samples displayed an indeterminate result and 10 gave a negative result (Table XIII).

Table XIII: Confirmation assays for EIA positive oral fluid specimens.

Country	Reactive EIA test	Reactive rapid test	Western Blot			Total No confirmed positive result
			Positive	Indeterminate	Negative	
Czech Rep.	11	10	0	0	1	10
Greece	15	12	3	0	0	15
Italy	52	34	14	0	4	48
Romania	17	9	6	1	1	15
Slovakia	23	15	7	1	0	22
Slovenia	24	18	2	0	4	20
Spain	66	53	13	0	0	66
Total	208	151	45	2	10	196

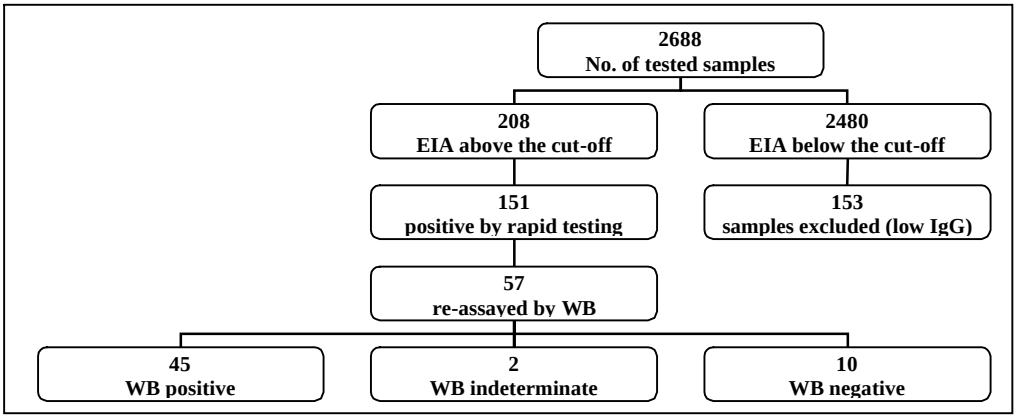
Of the samples tested negative by EIA, 153 out of 2480 (6.2%) were considered not valid and thus excluded from data analysis, because an insufficient IgG content. This resulted in a number of oral fluid (OF) samples considered appropriate for HIV testing of 2535 (Table XIV).

Table XIV: Oral fluid samples appropriate for HIV testing by IgG determination.

Country	No. oral fluid tested samples	No. oral fluid invalid samples	No. oral fluid valid samples
Czech Republic	418	31	387
Greece	178	28	150
Italy	500	11	489
Romania	398	53	345
Slovakia	396	10	386
Slovenia	399	10	389
Spain	399	10	389
Total	2688	153	2535

The results obtained in the survey by HIV oral fluid testing are summarized in Table XV.

Table XV: Overview of survey results on oral fluid samples.



The overall prevalence rate of HIV infection among tested MSM was 7.7% (196 out of 2535 men, 95% CI: 6.7-8.7%) (Table XVI).

Table XVI: Prevalence of HIV infection among MSM in South-Eastern Europe.

Country	Confirmed HIV positive OF samples	HIV prevalence (95% CI)
Czech Republic	10/387	2.6% (1-4.2%)
Greece	15/150	10% (5.2-14.8%)
Italy	48/489	9.8% (7.2-12.4%)
Romania	15/345	4.3% (2.2-6.4%)
Slovakia	22/386	5.7% (3.4-8%)
Slovenia	20/389	5.1% (2.9-7.3%)
Spain	66/389	17% (13.3-20.7%)
Total	196/2535	7.7% (6.7-8.7%)

A significant difference in HIV prevalence among MSM between Southern and Eastern European countries was observed 12.3% [10.3-14.3%] vs. 4.4% [3.4-5.4%]; $p<0.001$) (Fig. 17).

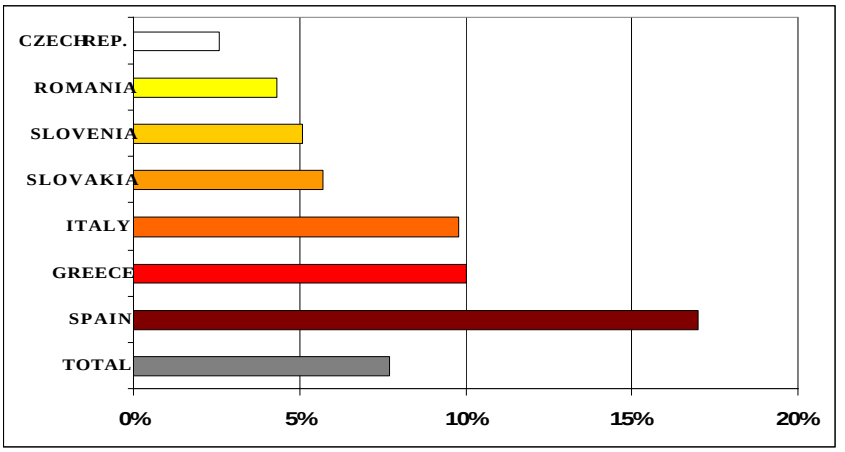


Fig. 17: HIV prevalence rates among MSM by country.

Spain had the highest proportion of HIV affected subjects (17%) followed by Greece (10%) and Italy (9.8%). On the other hand, the lowest HIV prevalence rates were found among MSM in Czech Republic (2.6%) and Romania (4.3%).

The age-specific HIV prevalence was analyzed and is shown in Fig. 18.

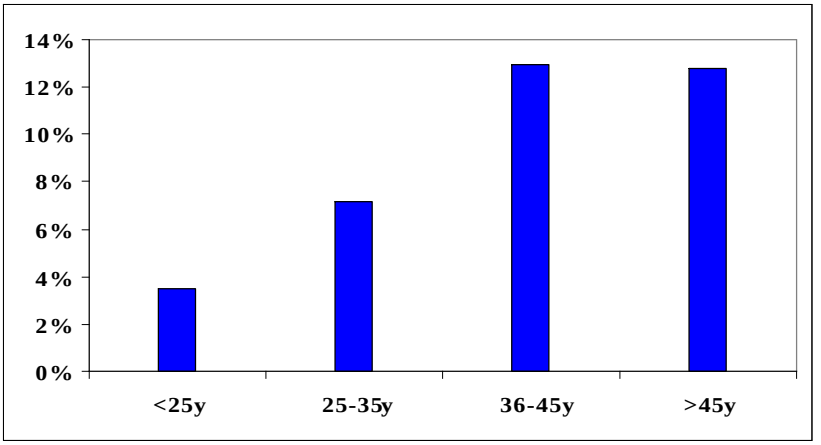


Fig.18: HIV prevalence by age.

People aged more than 25 years displayed a significantly higher HIV prevalence than other age-groups (3.5% vs 9.6%; $p<0.001$).

About a half of MSM with positive results on oral fluid testing were unaware of their real HIV status; the highest proportion was found in Slovenia (68%) and the lowest in Spain (38%) (Fig. 19).

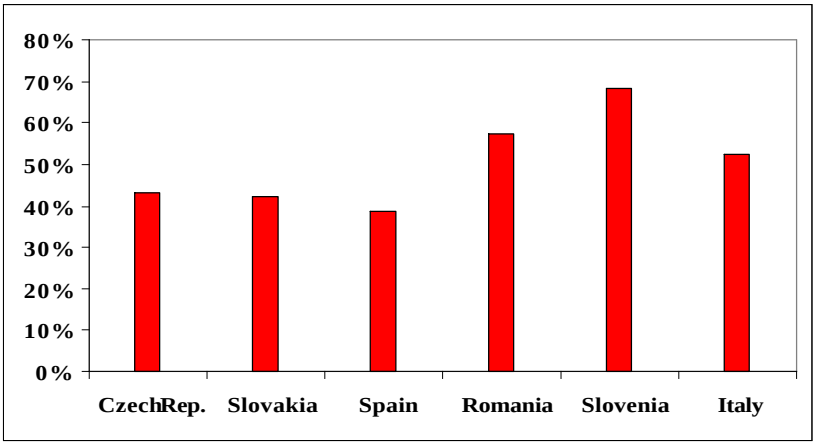


Fig.19: Proportion of HIV positive subjects unaware of their own serostatus.

Among the subjects tested HIV positive on oral fluid samples, those reporting the last negative HIV test within the previous 12 months were 52% (Fig. 20).

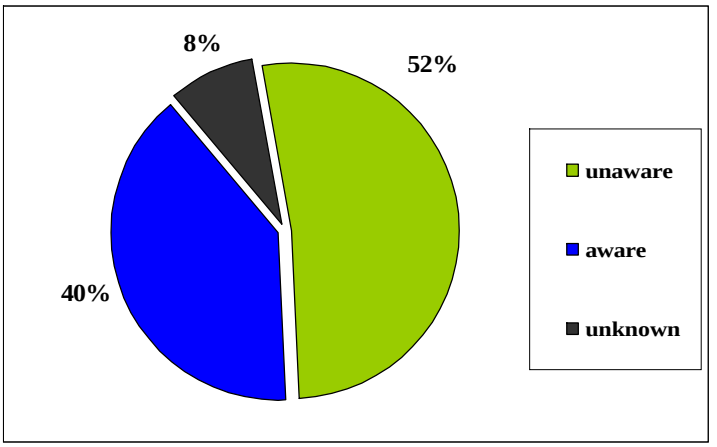


Fig. 20: Degree of HIV serostatus awareness among MSM tested HIV positive.

In addition, 16% of people who had an STI in the last 12 months were also HIV positive compared to 6.6% of people who didn't ($p<0.001$).

These data confirm that MSM represent a population at high risk for HIV transmission, where the awareness of real HIV serostatus lacks in nearly half cases of infected people.

DISCUSSION

1. VALIDATION STUDY

Since it has been reported that antibodies to HIV would be reliably detected in oral fluid samples of HIV infected patients, the present study evaluated the feasibility of an EIA oral fluid testing for anti-HIV antibodies in comparison to serum [99,112,116,119,138-142].

On validation phase, we obtained a striking concordance when paired serum and oral fluid samples were tested for anti-HIV antibodies by using Genscreen HIV 1/2 version 2 (k=0.98). The sensitivity and specificity of this assay on oral fluid samples was 98.5% and 100%, respectively.

These results suggested that Genscreen HIV 1/2 version 2 was a suitable assay for detection of antibodies to HIV-1/2 in oral fluids, thus finding an application in epidemiological surveys on HIV occurrence in high risk population.

There were 4 out of 263 specimens from HIV-seropositive subjects that were found discordant with regard to their EIA reactivity when serum and oral fluid were assayed; among them, 3 oral fluid samples were positive by WB, whereas 1 gave an indeterminate result.

Considering the number of false negative oral fluid results by country, 2 samples were from Slovenia (Se=97.2%), 1 from Greece (Se=98.6%) and 1 from Spain (Se=98.6%), whereas Czech Republic, Italy, Romania and Slovakia showed no discordant specimen results (Se=100%).

Reasons for these 4 false-negative results are difficult to pinpoint.

Although testing these samples for total IgG revealed a sufficient content in all four cases, it has to be considered that the quantification of IgG does not prove their intact function.

It is therefore conceivable that low specific or bacterial degraded IgG anti-HIV antibodies may have not reached the minimal threshold required to give positive OD in EIA [100,143]. Consistently with this hypothesis, the 5-fold amount of oral fluid used in Western blotting showed positive results in 3 over 4 samples.

A concomitant factor, previously observed in association with false negative reports on oral fluid testing, was the early initiation of antiretroviral treatment, due to its potential for decreasing specific serum antibody levels [144]. In this study, all these 4 patients were under antiretroviral therapeutic regimen.

The occurrence of false negative results may also be linked to the intrinsic limit of detection of the EIA assay, which is supported by the lower OD/CO ratio exhibited by concordantly negative oral fluid samples as compared to that found in false negative samples. However, by lowering the cut-off, the sensitivity of EIA testing on oral fluid specimens would improve, despite of specificity. Thus for epidemiological purposes an OD/CO ratio greater than 1 could be acceptable, to avoid the appearance of false positives.

We chose this oral fluid collection device, because it is cheap, safe and acceptable to individuals of all ages, and proved to yield high quality oral fluid samples for antibody testing in terms of total and specific antibody concentrations [110,117].

This device is designed to target the gum, the part of the oral cavity most likely to be rich in gingival crevicular fluid.

As opposed to blood, the collection of oral fluid is non-invasive, safe (as viral load is undetectable in oral fluid) and more cost effective. The combination of an adequate oral fluid collection device and a highly sensitive antibody detection assay may offer a good alternative to techniques based on serum for the surveillance of viral infections among high risk population. In this study, the oral fluid collection device used for detection for HIV antibodies was well-accepted, thus promoting high compliance among participants and proving to be ideally suited for field collection.

2. MULTI-CENTER SURVEY

With an estimated 40% increase in the proportion of HIV new infections due to MSM in Europe between 2003 and 2008, there is a need for surveillance interventions as efficient tools to monitor the HIV epidemic among this high-risk group [14,145].

We conducted a multi-center survey with the aim to establish the prevalence of HIV infection in MSM from seven Southern and Eastern European countries by using the previously validated EIA Genscreen HIV-1/2 version 2 (BIO-RAD) on oral fluid samples.

We found complete concordance between the reactive results from EIA oral fluid testing and confirmatory testing (rapid test or Western Blot) in 196 out of 208 cases (94.2%), whereas the rate of false positive results was 5.8%.

In our study, overall prevalence of anti-HIV antibodies among MSM was found to be 7.7% (196 out of 2535 men, 95% CI: 6.7-8.7%), a value much higher than that

estimated in European adult population (0.3%), suggesting that MSM are yet to have a major impact on the HIV epidemic [26].

In Spain, HIV prevalence rate among MSM was 17%, in concordance with prevalence data reported in a previous cross-sectional study conducted in Catalonia (18%) [15,16,26,146].

In Greece, where more than 50% of new HIV infections have been reported to be among MSM, the HIV prevalence rate has been high (10%) [15,147,148].

In Italy, the HIV prevalence observed in sentinel sites like Verona and Gorizia was 9.8%. This data associated with previous reports about relevant increase in HIV incidence observed among MSM in Rome from 1995 to 2003 (2.2% to 5%) show the spread of HIV infection among MSM to be continuing [149].

Similar to other studies carried out in Slovakia, a HIV prevalence of 5.7% among MSM was described [150,151]. In this country, MSM account for half newly diagnosed HIV infections [150].

The burden of HIV among MSM in Slovenia is disproportionally high (more than 70% cases of new infections) and exhibits an increasing trend (from 2.5 to 16.7 per million between 1999 to 2008) [150,152]. We estimated an HIV prevalence rate of 5.1%, that is higher than that reported from previous studies (2.1-3%) [16,29,152].

Romania and Czech Republic had HIV prevalence rates of 4.3 and 2.6%, respectively. A minimal proportion of all new HIV infections (5%) are due to MSM in Romania, where the leading route of transmission of infection is through heterosexual contact [16,150]. In Czech Republic, MSM account for 57% of all

reported HIV cases and HIV prevalence rates among MSM in previous reports ranged from 0.5% to 1.4% [16,28,150,153].

The association between age and anti-HIV prevalence was statistically significant in those 25 years or older as compared to younger subjects.

A history of STIs in the last 12 months was significantly associated to anti-HIV positivity ($p<0.001$). This is concordant with previous reports that genital ulcer diseases such as syphilis may increase the risk of HIV transmission, enhancing both the infectiousness of HIV and susceptibility to the virus [154,155].

With respect to access to HIV testing in the last 12 months, the highest percentage of tested MSM was found in Southern European countries as compared to Eastern countries, thus indicating a better voluntary counselling testing practice (65.8% vs. 44.9%; $p<0.001$).

About a half of MSM with positive results on oral fluid testing were unaware of their real HIV status; in particular, it raises concerns that the highest proportion of unaware HIV positive subjects were from Slovenia (68%), where HIV prevalence among MSM, the main driver of the epidemic in the country, has increased by 8 times in the last decade.

Despite of a negative HIV test report within the previous 12 months, more than half HIV positive subjects weren't aware of their real serostatus. This data suggest that these subjects had been infected during the last year and so relying only on a past negative test result may be an ineffective prevention strategy without additional behavioural interventions.

It is important to highlight that the overall HIV prevalence rate among MSM from Southern European countries was found to be significantly higher than that of Eastern countries, thus resembling higher rates of infection in adult population in Southern countries (0.3%) as compared to Eastern European region (0.1%) [16]. These data indicate that MSM have a one in eight odds of becoming infected with HIV infection in Southern Europe and a one in twenty-three odds in Eastern countries. Therefore, interventions focusing on reducing risk behaviour and preventing secondary transmission of HIV infection are urgently needed among this high risk group.

CONCLUSION

Our findings demonstrate for the first time that EIA Genscreen HIV-1/2 version 2 is a valuable tool for epidemiological surveillance of HIV infection, due to its reliability, convenience and user-friendliness.

In non-clinical settings, oral fluid-based linked HIV testing strategies would provide hard-to-reach population with access to testing services, thus increasing the awareness about their HIV serostatus. In addition, this allows for an estimation of prevalence rates of infection for surveillance purposes.

We conducted a large survey, where oral fluid-based testing was successfully used to obtain a one-time estimation of HIV prevalence from a large cohort of MSM at risk for infection in seven EU countries. The results of this multi-center study emphasize the high prevalence rate of HIV infected people among MSM, especially in Southern Europe and consequently the need for interventions aimed at encouraging behavioural changes among this high risk group.

REFERENCES

1. Joint United Nation Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO). AIDS epidemic update 2009. Geneva. UNAIDS 2009.
2. Kilmarx PH: Global epidemiology of HIV. Curr Opin HIV AIDS 2009; 4: 240-246.
3. Royce RA, Seña A, Cates jr, W, Cohen MS: Sexual transmission of HIV. N Engl J Med 1997; 336: 1072-1078.
4. Simon V, Ho DD, Karim QA: HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. Lancet 2006; 368: 489-504.
5. Mc Carthy M: HIV levels in mother's blood predicts risk of perinatal transmission. Lancet 1999; 354: 573.
6. Beyrer C: HIV epidemiology update and transmission factors: risks and risk contexts—16th international AIDS conference epidemiology plenary. Clin Infect Dis 2007; 44: 981-987.
7. European Centre for Disease Prevention and Control /WHO Regional Office for Europe. Surveillance report. HIV/AIDS surveillance in Europe 2008. Stockholm. ECDC 2009.
8. Hamers FF, Downs AM: HIV in Central and Eastern Europe. Lancet 2003; 361: 1035-1044.
9. CDC. HIV/AIDS Surveillance Report. Vol. 19; 2009.
10. CDC. Fact Sheet: The HIV/AIDS epidemic in the United States; September 2009.
11. CDC. Fact Sheet: Estimates of new HIV infections in the United States; August 2008.
12. Fenton KA: Changing epidemiology of HIV/AIDS in the United States: implications for enhancing and promoting HIV testing strategies. Clin Infect Dis 2007; 45: S213-S220.

13. Steinbrook R: The AIDS epidemic—a progress report from Mexico City. N Engl J Med 2008; 359: 885-887.
14. van de Laar MJW, Likatavicius G: HIV and AIDS in the European Union, 2008. Euro Surveill. 2009; 14.
15. Hamers FF, Downs AM: The changing face of the HIV epidemic in Western Europe: what are the implications for public health policies? Lancet 2004; 364: 83-94.
16. European Centre for Epidemiological Monitoring of AIDS (EuroHIV). HIV/AIDS surveillance in Europe. End-year report 2006. Saint-Maurice: Institut de Veille Sanitaire, 2007. No 75.
17. Suligoi B, Boros S, Camoni L, Lepore D, Santoro A: "Aggiornamento dei casi di AIDS e delle nuove diagnosi di infezioni da HIV in Italia al 31 Dicembre 2007". Not Ist Super Sanità 2008; 21: 3-23.
18. Suligoi B, Pavoni N, Borghi V, Branz F, Coppola N, Gallo G, Perucci CA, Piovesan C, Porta D, Rossetti G, Rezza G: "Epidemiologia dell'infezione da HIV in Italia". Epidemiol Prev 2003; 27: 73-79.
19. Suligoi B, Galli C: "Aggiornamenti sull'epidemiologia e la diagnosi di infezione da HIV". LigandAssay 2005; 10: 19-25.
20. Camoni L, Suligoi B: "Lo studio delle nuove diagnosi di infezione da HIV in Italia attraverso i sistemi di sorveglianza regionali: caratteristiche operative e valutazione". Ann Ist Super Sanità 2005; 41: 515-521.
21. Suligoi B, Boros S, Camoni L, Lepore D, Santoro A: "Aggiornamento delle nuove diagnosi di infezione da HIV al 31 Dicembre 2007 e dei casi di AIDS in Italia al 31 Dicembre 2008". Not Ist Super Sanità 2009; 22: 3-23.
22. Camoni L, Salfa MC, Regine V, Pasqualini C, Borghi V, Icardi G, Curtale F, Ferro A, Suligoi B: HIV incidence estimate among non-nationals in Italy. Eur J Epidemiol 2007; 22: 813-817.
23. Borghi V, Girardi E, Bellelli S, Angeletti C, Mussini C, Porter K, Esposito R: Late presenters in a HIV surveillance system in Italy during the period 1992-2006. J Acquir Immune Defic Syndr 2008; 49: 282-286.

24. Smith AD, Tapsoba P, Peshu N, Sanders EJ, Jaffe HW: Men who have sex with men and HIV/AIDS in sub-Saharan Africa. Lancet 2009; 374: 416-422
25. Baral S, Sifakis F, Cleghorn F, Beyrer C: Elevated risk for HIV infection among men who have sex with men in low- and middle- income countries 2000-2006: A systematic review. Plos Medicine 2007; 4.
26. van Griensven F, de Lind van Wijngaarden JW, Baral S, Grulich A: The global epidemic of HIV infection among men who have sex with men. Curr Opin HIV AIDS 2009; 4: 300-307.
27. CDC. Fact Sheet: HIV and AIDS among gay and bisexual men; August 2009.
28. European Centre for Disease Prevention and Control /WHO Regional Office for Europe. Surveillance report. HIV/AIDS surveillance in Europe 2007. Stockholm. ECDC 2008.
29. Likatavicius G, Klavs I, Devaux I, Alix J, Nardone A: An increase in newly diagnosed HIV cases reported among men who have sex men in Europe, 2000-6: implications for a European public health strategy. Sex Transm Infect 2008; 84: 499-505.
30. CDC: Revised recommendations for HIV testing of adults, adolescents and pregnant women in health-care settings. MMWR 2006; 55:1-17.
31. Jaffe HW, Valdiserri RO, De Cock KM: The re-emerging HIV/AIDS epidemic in men who have sex with men. JAMA 2007; 298: 2412-2414.
32. Levy JA: “Scoperta, struttura, eterogeneità ed origine di HIV”. In: Levy JA ed. HIV patogenesi dell’AIDS, Piccin, 2007, pp 253-281.
33. Nakai M, Goto T: Ultrastructure and morphogenesis of Human Immunodeficiency Virus. J Electron Microsc 1996; 45: 247-257.
34. Turner BG, Summers MF: Structural biology of HIV. J Mol Biol 1999; 285: 1-32.
35. Cullen BR: Regulation of HIV-1 gene expression. AIDS 1995; 9: S19-S32.
36. Reeves JD, Doms RW: Human immunodeficiency virus type 2. J Gen Virol 2002; 83: 1253-1265.

37. Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, Michael SF, Cummins LB, Arthur LO, Peeters M, Shaw GM, Sharp PM, Hahn BH: Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature 1999; 397: 436-441.

38. Nerrienet E, Santiago ML, Foupouapouognigni Y, Bailes E, Mundy NI, Njinku B, Kfutwah A, Muller-Trutwin MC, Barre-Sinoussi F, Shaw GM, Sharp PM, Hahn BH, Ayouba A: Simian Immunodeficiency Virus infection in wild-caught chimpanzees from Cameroon. J Virol 2005; 79: 1312-1319.

39. Whittle HC, Ariyoshi K, Rowland-Jones SL: HIV-2 and T cell recognition. Curr Opin Immunol 1998; 10: 382-387.

40. Barin F, Cazein F, Lot F, Pillonel J, Brunet S, Thierry D, Damond F, Brun-Vezenet F, Desenclos JC, Semaille C: Prevalence of HIV-2 and HIV-1 group O infections among new HIV diagnoses in France: 2003-2006. AIDS 2007; 21:2351-2353.

41. Valadas E, Franca L, Sousa S, Antunes F: 20 years of HIV-2 infection in Portugal: trends and changes in epidemiology. CID 2009; 48:1166-1167.

42. de Silva TI, Cotton M, Rowland-Jones SL: HIV-2: the forgotten AIDS virus. Trends Microbiol 2008; 16: 588-595.

43. Levy JA: HIV pathogenesis: 25 years of progress and persistent challenges. AIDS 2009; 23: 147-160.

44. McCutchan FE: Global epidemiology of HIV. J Med Virology 2006; 78: S7-S12.

45. Geretti AM: HIV-1 subtypes: epidemiology and significance for HIV management. Curr Opin Infect Dis 2006; 19: 1-7.

46. Buonaguro L, Tornasello ML, Buonaguro FM: Human Immunodeficiency Virus type 1 subtype distribution in the worldwide epidemic: pathogenic and therapeutic implications. J Virol 2007; 81: 10209-10219.

47. Taylor BS, Sobieszczyk ME, McCutchan FE, Hammer SM: The challenge of HIV-1 subtype diversity. N Engl J Med 2008; 358: 1590-1602.

48. Holguin A, Gutierrez M, Portocarrero N, Rivas P, Baquero M: Performance of OraQuick Advance® Rapid HIV-1/2 Antibody Test for detection of

antibodies in oral fluid and serum/plasma in HIV-1^{+}subjects carrying different HIV-1 subtypes and recombinant variants. J Clin Virol 2009; 45: 150-152.

49. Cairns JS, D'Souza MP: Chemokines and HIV-1 second receptors: the therapeutic connection. Nature Medicine 1998; 4: 563-568.
50. Tang H, Kuhen KL, Wong-Staal F: Lentivirus replication and regulation. Ann Rev Genetics 1999; 33: 133-170.
51. Schacker T, Collier AC, Hughes J, Shea T, Corey L: Clinical and epidemiologic feature of primary HIV infection. Ann Int Med 1996; 125: 257-264.
52. Devare SG: Early diagnosis of HIV infection. J Med Virol 2007; 79: S11-S15.
53. Pantaleo G, Graziosi C, Demarest JF, Butini L, Montroni M, Fox CH, Orestein JM, Kotler DP, Fauci AS: HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 1993; 362: 355-358.
54. Mindel A, Tenant-Flowers M: Natural history and management of early HIV infection. BMJ 2001; 322: 1290-1293.
55. Piot P, Colebunders R: Clinical manifestations and the natural history of HIV infection in adults. West J Med 1987; 147: 709-712.
56. CDC. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescent and adults. MMWR 1992; 41.
57. Markovitz DM: Infection with the Human Immunodeficiency Virus type 2. Ann Int Med 1993; 118: 211-218.
58. Guedin M, Damond F, Braun J, Taieb A, Lemmee V, Plantier JC: Differences in proviral DNA load between HIV-1- and HIV-2- infected patients. AIDS 2008; 22: 457-468.
59. Michel PAT, Balde C, Roussilhon C, Aribot G, Sarthou JL, Gougeon ML: Reduced immune activation and T cell apoptosis in human immunodeficiency virus type 2 compared with type 1: correlation of T cell

apoptosis with beta 2 microglobulin concentration and disease evolution. J Infect Dis 2000; 181: 64-75.

60. De Cock K, Adjorlolo G, Ekpini E, Sibailly T, Kouadio J, Maran M, Brattegaard K, Vetter KM, Doorly R, Gayle HD: Epidemiology and transmission of HIV-2: why there is no HIV-2 pandemic. JAMA 1993; 270: 2083-2086.
61. Bjorling E, Scarlatti EG, Von Gegerfelt A, Albert J, Biberfeld G, Chiodi F, Norby E, Fenyo EM: Autologous neutralizing antibodies prevail in HIV-2 but not in HIV-1 infection. Virology 1993; 193: 528-530.
62. Rowland-Jones S: Protective immunity against HIV infection: lesson from HIV-2 infection. Future Microbiol 2006; 1: 427-433.
63. Ahmed RK, Norrgren H, da Silva Z, Blaxhult A, Fredriksson EL, Biberfeld G, Andersson S, Thorstensson R: Antigen-specific b-chemokine and CD8 T-cell noncytotoxic antiviral activity in HIV-2 infected individuals. Scand J Immunol 2005; 61: 63-71.
64. Coombs RW: Clinical laboratory diagnosis of HIV-1 and use of viral RNA to monitor infection. In: Holmes KK, Sparling PF, Stamm WE et al., eds. Sexually Transmitted Diseases 4th edn, 2008, Mc Graw-Hill, pp1329-1347.
65. Busch MP, Satten GA: Time course of viremia and antibody seroconversion following human immunodeficiency virus exposure. Am J Med 1997; 102: 117-124.
66. Marquez C, Zetola NM, Klausner JD: HIV testing: an update. MLO Med Lab Obs 2008; 40:12-21.
67. Costantine NT, Zink H: HIV testing technologies after two decades of evolution. Indian J Med Res 2005; 121: 519-538.
68. Branson BM: State of art for diagnosis of HIV infection. Clin Infect Dis 2007; 45: S221-225.
69. Troung HM, Klausner JD: Diagnostic assays for HIV-1 infection. MLO Med Lab Obs 2004; 36:12-20.
70. Pilcher CD, Fiscus SA, Nguyen TQ, Foust E, Wolf L, Williams D, Ashby R, O'Dowd JO, McPherson JT, Stalzer B, Hightow L, Miller WC, Eron jr

JJ, Cohen MS, Leone PA: Detection of acute infections during HIV testing in North Carolina. N Engl J Med 2005; 352: 1873-1883.

71. Costantine NT, Kabat W, Zhao RY: Update on the laboratory diagnosis and monitoring of HIV infection. Cell Research 2005; 15: 870-876.
72. Luft S, Seme K, Poljak M: Laboratory diagnosis of human immunodeficiency virus infection. Acta Dermatoven APA 2004; 13: 43-49.
73. Mylonakis E, Paliou M, Lally M, Flanigan TP, Rich JD: Laboratory testing for infection with the Human Immunodeficiency Virus: established and novel approaches. Am J Med 2000; 119: 568-575.
74. Fearon M: The laboratory diagnosis of HIV infections. Can J Dis Med Microbiol 2005; 16: 26-30.
75. Dax EM, Arnott A: Advances in laboratory testing for HIV. Pathology 2004; 36: 551-560.
76. Griffith BP, Campbell S, Mayo DR: Human Immunodeficiency Viruses. In: Murray PR, Baron EJ, Jorgensen JH, et al., eds. Manual of clinical microbiology, 9th edn Washington: ASM Press, 2007, pp.1308-1329.
77. Johnson JE: Detection of Human immunodeficiency virus type 1 antibody by using commercially available whole-cell viral lysate, synthetic peptides and recombinant protein enzyme immunoassay systems. J Clin Microbiol 1992; 30: 216-218.
78. Branson BM: point-of-care rapid tests for HIV antibodies. J Lab Med 2003; 27: 288-295.
79. Donovan BJ, Rublein JC, Leone PA, Pilcher CD: HIV infection: point-of-care testing. Ann Pharmacother 2004; 38: 670-676.
80. Granade TC: Use of rapid HIV antibody testing for controlling the HIV pandemic. Expert Rev Anti Infect Ther 2005; 3: 957-969.
81. Branson B: Current HIV epidemiology and revised recommendations for HIV testing in health-care settings. J Med Virol 2007; 79: S6-S10.
82. Paredes CF, Tellez I, del Rio C: Rapid HIV testing: a review of the literature and implications for clinician. Curr HIV/AIDS Rep 2006; 3: 159-165.

83. Iqbal HS, Balakrishnan P, Murugavel KG, Suniti S: Performance characteristics of a new rapid immunochromatographic test for the detection of antibodies to Human Immunodeficiency Virus (HIV) types 1 and 2. J Clin Lab Anal 2008; 22: 178-185.
84. WHO: WHO criteria for interpreting results from Western Blot assay for HIV-1, HIV-2, HTLV-I/II. Wkly Epidemiol Rec 1990; 37: 281-283.
85. CDC interpretive criteria used to report Western Blot result for HIV-1 antibody testing-United States. MMWR 1991;41: 692-695.
86. Weber B: Screening of HIV infection: role of molecular and immunological assays. Expert Rev mol Diagn 2006; 6: 399-411.
87. Stekler J, Maenza J, Stevens CE, Swenson PD, Coombs RW, Wood RW, Campbell MS, Nickle DC, Collier AC, Golden MR: Screening for acute HIV infection: lesson learned. Clin Infect Dis 2007; 44: 459-461.
88. Fiebig EW, Wright DJ, Rawal BD, Garrett PE, Schumacher RT, Peddada L, Heldebrant C, Smith R, Conrad A, Kleinmam SH, Busch MP: Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. AIDS 2003; 17: 1871-1879.
89. Hetcht FM, Busch MP, Rawal B, Webb M, Rosemberg E, Swanson M, Chesney M, Anderson J, Levy J, Kahn JO: Use of laboratory tests and clinical symptoms for identification of primary HIV infection. AIDS 2002; 16: 1119-1129.
90. Rich JD, Merriman NA, Mylonakis E, Greenough TC, Flanigan TP, Mady BJ, Carpenter CCJ: Misdiagnosis of HIV infection by HIV-1 plasma viral load testing: a case series. Ann Intern Med 1999; 130: 37-39.
91. World Health Organization-UNAIDS. Guidelines for measuring national HIV prevalence in population-based surveys. Geneva: WHO/UNAIDS, 2005.
92. World Health Organization-UNAIDS. Guidelines for using HIV testing technologies in surveillance. Geneva: WHO/UNAIDS, 2001.

93. World Health Organization. Revision of HIV testing strategies for surveillance. Wkly Epidemiol Rec 2006; 48: 461-464.
94. Respess RA, Rayfield MA, Dondero TJ: Laboratory testing and rapid HIV assays: applications for HIV surveillance in hard-to-reach populations. AIDS 2001; 15: S49-S59.
95. UNAIDS-WHO. Revised recommendations for the selection and use of HIV antibody tests. Wkly Epidemiol Rec 1997; 72: 81-88.
96. Kaufman E, Lamster IB: The diagnostic applications of saliva- a review. Crit Rev Oral Biol Med 2002; 13: 197-212.
97. Malamud D: Saliva as a diagnostic fluid. BMJ 1992; 305: 207-208.
98. Spielberg F, Branson BM, Goldbaum GM, Lockhart D, Kurth A, Rossini A, Wood RW: Choosing HIV counselling and testing strategies for outreach settings.J Acquir Immun Defic Syndr 2005; 38: 348-355.
99. Parry JV, Perry KR, Mortimer PP: Sensitive assays for viral antibodies in saliva: an alternative to tests on serum. Lancet 1987; 71-75.
100. Malamud D: Oral diagnostic testing for detecting human immunodeficiency virus-1 antibodies: a technology whose time has come. Am J Med 1997; 102: S9-S14.
101. Major CJ, Read SE, Coates RA, Francis A, McLaughlin BJ, Millson M, Shepherd F, Fanning M, Calzavara L, MacFadden D, Johnson JK, O'Shaughnessy MV: Comparison of saliva and blood for human immunodeficiency virus prevalence testing. J Infect Dis 1991; 163: 699-702.
102. De Cock L, Hutse V, Verhaegen E, Quoilin S, Vandenberghe H, Vranckx R: Detection of HCV antibodies in oral fluid. J Virol Methods 2004; 122: 179-183.
103. Amado LA, Villar LM, de Paula VS, de Almeids AJ, Gaspar AMC: Detection of hepatitis A,B,C virus specific antibodies using oral fluid for epidemiological studies. Mem Inst Oswaldo Cruz 2006; 101: 149-155.

104. Kremer JR, Muller CP: Evaluation of commercial assay detecting specific immunoglobulin G in oral fluid for determining measles immunity in vaccines. Clin Diagn Lab Immunol 2005; 12: 668-670.
105. Thieme T, Piacentini S, Davidson S, Steingart K: Determination of measles, mumps and rubella immunization status using oral fluid samples. JAMA 1994; 272: 219-221.
106. Shoeman RL, Pottathil R, Metroka C: Antibodies in saliva. N Engl J Med 1989; 320: 1145-1146.
107. Madar R, Straka S, Baska T: Detection of antibodies in saliva—an effective auxiliary method in surveillance of infectious diseases. Bratisl Lek Listy 2002; 103: 38-41.
108. Samaranayake L: Saliva as a diagnostic fluid. Int Dent J 2007; 57: 295-299.
109. Scully C: HIV topic update: salivary testing for antibodies. Oral Diseases 1997; 3: 212-215.
110. Vyse AJ, Cohen BJ, Ramsay ME: A comparison of oral fluid collection devices for use in the surveillance of virus diseases in children. Public Health 2001; 115: 201-207.
111. Tamashiro H, Constantine NT: Serological diagnosis of HIV infection using oral fluid samples. Bull W.H.O. 1994; 72: 135-143.
112. Emmons W: Accuracy of oral specimen testing for Human Immunodeficiency Virus. Am J Med 1997; 102: S15-S20.
113. George JR, Fitchen JH: Future applications of oral fluid specimen technology. Am J Med 1997; 102: S21-S25.
114. Hodinka RL, Nagashunmugam T, Malamud D: Detection of Human Immunodeficiency Virus antibodies in oral fluids. Clin Diagn Lab Immunol 1998; 5: 419-426.
115. McKie A, Vyse A, Maple C: Novel methods for the detection of microbial antibodies in oral fluid. Lancet 2002; 2: 18-24.
116. Gallo D, George JR, Fitchen JH, Goldstein AS, Hindahl MS: Evaluation of a system using oral mucosal transudate for HIV-1 antibody screening and confirmatory testing. JAMA 1997; 277: 254-258.

117. Nokes DJ, Enquselassie F, Vyse A, Nigatu W, Cutts FT, Brown DWG: An evaluation of oral-fluid collection devices for the determination of rubella antibody status in a rural Ethiopian community. Trans R Soc Trop Med Hyg 1998; 92: 679-685.
118. Archibald DW, Zon LI, Groopman JE, McLane MF, Essex ME: Antibodies to Human T Lymphotrophic Virus type III (HTLV-III) in saliva of acquired immunodeficiency syndrome (AIDS) patients and in persons at risk for AIDS. Blood 1986; 67:831-834.
119. van den Akker R, van den Hoek AR, van den Akker WMR, Kooy H, Vijge E, Roosendaal G, Coutinho RA, van Loon AM: Detection of HIV antibodies in saliva as a tool for epidemiological studies. AIDS 1992; 6: 953-957.
120. Behets FM, Edidi B, Quinn TC, Atikala L, Bishagara K, Nzila N, Laga M, Piot P, Ryder RW, Brown CC: Detection of salivary HIV-1 specific IgG antibodies in high risk population in Zaire. J Acquir Immune Defic Syndr 1991; 4: 183-187.
121. Granade TC, Phillips SK, Parekh B, Pau CP, George JR: Oral fluid as a specimen for detection and confirmation of antibodies to Human Immunodeficiency Virus type 1. Clin Diagn Lab Immunol 1995; 2: 395-399.
122. Hunt AJ, Connell J, Christofinis G, Parry JV, Weatherburn P, Hickson FCI, Coxon APM, Davies PM, McManus YJ, Sutherland S: The testing of saliva samples for HIV-1 antibodies: reliability in a non-clinic setting. Genitourin Med 1993; 69: 29-30.
123. Thongcharoen P, Wasi C, Louisrirotchanakul S, Parry J, Connell J, Mortimer P: Immunoglobulin G antibody capture enzyme-linked immunosorbent assay: a versatile assay for detection of anti-human immunodeficiency virus type 1 and 2 antibodies in body fluids. J Clin Microbiol 1992; 30: 3288-3289.
124. Luo N, Kasolo F, Ngwenya BK, du Port HL, Zumla A: Use of saliva as an alternative to serum for HIV screening in Africa. S Afr Med J 1995; 85: 156-157.

125. Johnson AM, Parry JV, Best SJ, Smith AM, de Silva M, Mortimer PP: HIV surveillance by testing saliva. AIDS 1988; 2: 369-371.
126. Crofts N, Nicholson S, Coghlan P, Gust ID: Testing of saliva for antibodies to HIV-1. AIDS 1991; 5: 561-563.
127. Granade TC, Phillips SK, Parekh B, Gomez P, Kitson-Piggott W, Oleander H, Mahabir B, Charles W, Lee-Thomas S: Detection of antibodies to Human Immunodeficiency Virus type 1 in oral fluids: a large-scale evaluation of immunoassay performance. Clin Diagn Lab Immunol 1998; 5: 171-175.
128. Chohan BH, Lavreys L, Mandaliya KN, Kreiss JK, Bwayo JJ, Ndinya-Achola JO, Martin Jr HL: Validation of a modified commercial enzyme – linked immunoassay for detection of Human Immunodeficiency Virus type 1 immunoglobulin G antibodies in saliva. Clin Diagn Lab Immunol 2001; 8: 346-348.
129. Soto-Ramirez LE, Hernandez-Gomez L, Sifuentes-Osornio J, Barriga-Angulo G, Duarte De Lima D, Lopez-Portillo M, Ruiz-Palacios GM: Detection of specific antibodies in gingival crevicular transudate by enzyme-linked immunosorbent assay for diagnosis of Human immunodeficiency Virus type 1 infection. J Clin Microbiol 1992; 30: 2780-2783.
130. Solomon S, Kumarasamy N, Martin SP, Pandian R, Sathiamoorthy K, Thyagarajan SP: Analysis of risk factors and evaluation of HIV testing in saliva and blood samples. J Infection 1997; 34: 85-91.
131. Stark K, Warnecke C, Brinkmann V, Gelderblom HR, Bienzle U, Pauli G: Sensitivity of HIV antibody detection in saliva. Med Microbiol Immunol 1993; 182: 147-151.
132. Frerichs RR, Silarug N, Eskes N, Pagcharoenpol P, Rodklai A, Thangsupachai S, Wongba C: Saliva-based antibody testing in Thailand AIDS 1994; 8: 885-894.

133. Fitzgibbons D, Barriga G, Seymour E, Stramer S: HIV antibody EIA and Western Blot assay results on paired serum and saliva specimens. Ann NY Acad Sci 1993; 694: 314-316.
134. Pai NP, Joshi R, Dogra S, Taksande B, Kalantri SP, Pai M, Narang P, Tulsky JP, Reingold AL: Evaluation of diagnostic accuracy, feasibility and client preference for rapid oral fluid-based diagnosis of HIV infection in rural India. PloS ONE 2007; 4.
135. Greenwald JL, Burstein GR, Pincus J, Branson B: A rapid review of rapid HIV antibody tests. Curr Infect Dis Rep 2006; 8: 125-131.
136. Jafa K, Patel P, Mackellar DA, Sullivan PS, Delaney KP, Sides TL, Newman AP, Paul SM, Cadoff EM, Martin EG, Keenan PA, Branson BM: Investigation of false positive results with an oral fluid rapid HIV-1/2 antibody test. PloS ONE 2007; 2.
137. Delaney KP, Branson BM, Uniyal A, Kerndt PR, Keenan PA, Jafa K, Gardner AD, Jamieson DJ, Bulterys M: Performance of an oral fluid rapid HIV-1/2 test: experience from four CDC studies. AIDS 2006; 20: 1655-1660.
138. Martinez P, Rodriguez-Torres A, Ortiz de Lejarazu R, Montoya A, Martin JF, Eiros JM: Human Immunodeficiency Virus antibody testing by enzyme-linked fluorescent and western blot assays using serum, gingival-crevicular transudate, and urine samples. J Clin Microbiol 1999; 37: 1100-1106.
139. Ettiegne-Traore V, Ghys PD, Maurice C, Hoyi-Adonsou YM, Soroh D, Adom ML, Teurquetil MJ, Diallo MO, Laga M, Greenberg AE: Evaluation of an HIV saliva test for the detection of HIV-1 and HIV-2 antibodies in high-risk populations in Abidjan, Cote d'Avoire. Int J STD AIDS 1998; 9: 173-174.
140. King SD, Wynter SH, Bain BC, Brown WA, Johnston JN, Delk AS: Comparison of testing saliva and serum for detection of antibody to Human Immunodeficiency Virus in Jamaica, West Indies. J Clin Virol 2000; 19: 157-161.

141. Hamers RL, de Beer IH, Kaura H, van Vugt M, Caparos L, Rinke de Wit TF: Diagnostic accuracy of 2 oral fluid –based test for HIV surveillance in Namibia. J Acquir Immune Defic Syndr 2008; 48: 116-118.
142. Alemnji GA, Ngulefac GA, Ndumbe PM, Asonganyi T: Field evaluation of Calypte's AWARE ™ blood serum plasma (BSP) and oral mucosal transudate (OMT) rapid tests for detecting antibodies to HIV-1 and 2 in plasma and oral fluid. The Open AIDS J 2009; 3: 14-18.
143. Mortimer PP, Parry JV: The use of saliva for viral diagnosis and screening. Epidemiol Infect 1988; 101:197-201.
144. O'Connell RJ, Merritt TM, Malia JA, Vancott TC, Dolan MJ, Zawha H, Bradley WP, Branson BM, Michael NL,De Witt CC: Performance of the OraQuick Rapid antibody test for diagnosis of human immunodeficiency virus type 1 infection in patients with various levels of exposure to highly active antiretroviral therapy. J Clin Microbiol 2003; 41: 2153-2155.
145. European Centre for Epidemiological Monitoring of AIDS (EuroHIV): MSM in Europe. Saint-Maurice: Institut de Veille sanitaire 2007.
146. Folch C, Casabona J, Muñoz R, Gonzales V, Zaragoza K: Increase in the prevalence of HIV and in associated risk behaviours in men who have sex with men: 12 years of behavioural surveillance surveys in Catalonia (Spain). Gac Sanit 2010; 24: 40-46.
147. Tsantes A, Nikolopoulos G, Masgala A, Paraskeva D: Assessing the secular trends in the transmission of HIV in Greece. Sex Transm Infect 2005; 81: 230-232.
148. Nikolopoulos G, Arvanitis M, Masgala A, Paraskeva D: Migration and HIV epidemic in Greece. Eur J Pubic Health 2005; 15: 296-299.
149. Giuliani M, Di Carlo A, Palamara G, Dorrucci M, Latini A, Prignano G, Stivali F, Rezza G: Increased HIV incidence among men who have sex with men in Rome. AIDS 2005; 19: 1429-1430.
150. Bozicevic I, Voncina L, Zigrovic L, Munz M, Lazarus JV: HIV epidemics among men who have sex with men in central and eastern Europe. Sex Transm Infect 2009; 85: 336-342.

151. Stanekova D, Habekova M, Wimmerova S, Gramblickova I: HIV infection and sexual behaviour among homosexual and bisexual men in Bratislava. Cent Eur J Public Health 2000; 8: 172-175.
152. Klavs I, Bergant N, Kastelic Z, Lamut A, Kustec T: Disproportionate and increasing burden of HIV infection among men who have sex with men in Slovenia: surveillance data for 1999-2008. Euro Surveill 2009; 14.
153. Bruckova M, Maly M, Vandasova J, Maresova M: HIV/AIDS in the Czech Republic, 2006. Euro Surveill 2007; 12.
154. Karp G, Schlaeffer F, Jotkowitz A, Riesenberg K: Syphilis and HIV co-infection. Eur J Int Med 2009; 20: 9-13.
155. Zetola NM, Klausner JD: Syphils and HIV infection: an update. Clin Infect Dis 2007; 44: 1222-1228.