Suggested strategies for the laboratory diagnosis of HIV infection in Italy

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Summary. HIV/AIDS surveillance data indicate that, in 2008, approximately one-fourth of all HIV infections in adults remain undiagnosed in Italy and that close to 60% of AIDS diagnosed individuals discovered their seropositivity at the diagnosis of AIDS. Late diagnosis of HIV infection is associated with increased mortality and morbidity and increased cost to healthcare services. From a public health perspective, knowledge of HIV status is associated with a reduction in risk behaviour. Thus, a routine screening for HIV infection is important for both a better prognostic outcome, and control of HIV spreading in the population. In Italy there are not shared guidelines for the laboratory diagnosis. In this paper, we suggest two algorithms that can be adopted for the diagnosis of HIV infection in individuals undergoing HIV testing.

Key words: HIV, diagnosis, guideline, algorithms, Italy.

Riassunto (*Proposta di strategie per la diagnosi di laboratorio dell'infezione da HIV in Italia*). Dati di sorveglianza stimano che, nel 2008, in Italia, erano circa un quarto le infezioni da HIV non diagnosticate e che quasi il 60% delle persone con AIDS ha scoperto la propria sieropositività alla diagnosi di AIDS. La diagnosi tardiva di infezione da HIV è associata a incrementata mortalità e morbidità ed aumentati costi per i servizi sanitari. Dal punto di vista della sanità pubblica, una diagnosi precoce di infezione da HIV è associata alla riduzione dei comportamenti a rischio di infezione. Pertanto, è importante potenziare lo screening sierologico per la diagnosi di laboratorio di infezione da HIV, per migliorare la prognosi e controllare più efficacemente la diffusione del virus, riducendo, così, i costi sanitari. In Italia non esistono linee guida per la diagnosi di laboratorio dell'infezione da HIVcondivise a livello nazionale. In questo articolo suggeriamo due possibili algoritmi per la diagnosi di infezione da HIV in individui che desiderano sottoporsi al test per HIV.

Parole chiave: HIV, diagnosi, linee guida, algoritmi, Italia.

INTRODUCTION

The introduction of highly active antiretroviral therapy (HAART) has led to a significant reduction in AIDS-related morbidity and mortality. Although guidelines for HIV antiretroviral therapy agree that the optimal time to start therapy for an asymptomatic patient with a CD4+ count of more than 350 cells per microliter is unknown, there is a general consensus to start therapy before AIDS-related symptoms become evident [1-4].

Italian surveillance estimates indicate that approximately one-fourth of all HIV infections in adults remain undiagnosed [5] and that, in 2008, approximately 60% per cent of AIDS diagnosed individuals discovered their seropositivity at the diagnosis of AIDS. Late diagnosis of HIV infection has been associated with increased mortality and morbidity, impaired response to HAART and increased costs to healthcare services [6]. Furthermore, from a pub-

lic health perspective, knowledge of HIV status is associated with a reduction in risk behaviour [7]. Thus, improvement of the frequency and the efficiency of routine screening for HIV infection may result, for the patient, in a better outcome of the disease and, from a public health perspective, in a better and cost effective control of HIV spreading in the population.

HIV testing strategies are different, depending on the testing purposes. HIV testing is essential in minimising HIV infection by transfusion of HIV-infected donated blood. Furthermore, testing for HIV infection can be used in sentinel surveys in specific populations [8]. Finally, HIV testing is necessary for the individual who wish to know his/her serostatus for HIV infection.

In Italy, no updated and consensus guidelines regulate the way how the diagnosis of HIV infection is performed. Only occasional indications, limited to

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peculiar situations have been provided in the past [9, 10]. In addition, more sensitive and specific assays have been developed in the last years that justify a critic revision of procedures for laboratory diagnosis of HIV infection.

We assert the opportunity that consensus guidelines for the laboratory diagnosis of HIV infection are adopted in Italy and propose, here, a strategy for HIV testing, limiting our discussion to HIV testing in individuals who wish to be tested for HIV infection.

TESTING FOR THE DIAGNOSIS OF HIV INFECTION

The most widely used tests for the laboratory diagnosis of HIV infection are the enzyme immuno assays (EIA) for the detection of HIV antibodies. Since the availability of the first EIA in 1985, these assays have undergone several enhancements, in both their formulation, and the assay dynamics and detection technologies. Almost all tests currently available and employed in Italy are either third-generation assays, that allow the recognition of HIV-specific antibodies of all Ig classes, or fourth-generation assays, that are able to detect both HIV antibodies and the p24 Gag antigen, simultaneously. Furthermore, nearly all assays employ signal amplification techniques (fluorescence, chemioluminescence) to further enhance sensitivity and are run on automated platforms, and thus are the most appropriate for testing large numbers of specimens on daily basis, since they are easy to perform, require less manipulation of the samples and are cost-effective (reviewed in [11]). When used in countries with a low HIV prevalence (less than 1%), such as Italy, these very sensitive assays have a very high negative predictive value (NPV) [8]. Thus, while in these countries a negative test result indicates lack of infection with a probability very close to 100%, a positive result could indicate, in rare occasions, presence of HIV infection in a non HIV-infected individual. For this reason, it is essential to confirm a first positive result obtained by EIA by using a second confirmatory test. Conversely, a negative result does not need to be confirmed, and no follow up is required, unless the person reports behaviours at risk for HIV infection and/or acquisition of sexually-related diseases.

The most commonly used confirmatory tests are the western blot (WB) and the line immuno assays (LIA) (reviewed in [11]). The use of WB can be expensive; however, in low HIV prevalence countries, like Italy [8], the impact of confirmatory assays on costs should not be so high, since a relative low number of positive EIA to confirm in WB is expected. Nevertheless, WB can produce a large number of indeterminate results and interpretation of results is subjective. Similar assays, such as LIA, based on recombinant proteins and/or synthetic peptides capable of detecting antibodies to specific HIV-1 and/ or HIV-2 proteins, can be used because, in general,

these assays produce fewer indeterminate results, as compared to WB. However, also with these tests, interpretation of results depends subjectively on the operator.

SPECIMENS

Venous blood serum without anticoagulant is preferred for HIV testing. In case RNA testing is foreseen, blood must be collected with anticoagulant (ethylene-diamine-tetraacetic acid, EDTA) or Na citrate. Heparin must be avoided, since it may interfere with polymerase chain reaction. Venous blood samples should be centrifuged in order to separate plasma/serum from blood cells and/or clot. When possible, it is advisable not to make aliquots of samples, in order to avoid labelling mistakes. However, it also must be taken into account that red blood cells haemolysis can occur, which may interfere and provide false positive results [12]. Therefore, separation of plasma/serum from blood cells and clot within few hours from bleeding is necessary. With this in mind, it is recommended to perform the initial screening test from the original primary tube, immediately after collection and, in case of a positive result or when haemolysis is visible, to repeat the test starting from the aliquot tube after clearing plasma/ serum by centrifugation. Testing with samples other than venous blood, such as finger prick blood, oral fluid or urine is feasible only with rapid tests, which, at the moment, are not as sensitive and specific as the conventional EIA screening and confirmatory tests (reviewed in [11]). Rapid tests can be useful in certain settings, such as needlephobic patients, for epidemiological purposes [8], for screening children where referral to a paediatric phlebotomist is impractical [13], and when the need for an immediate HIV result is crucial for decision making.

According to European regulations, tested plasma/serum samples should be stored as long as possible, however never less than 48 hours after the report has been released, since additional tests may be requested [14].

KIT AND LABORATORIES REQUIREMENTS FOR HIV TESTING

Only Conformité Européenne (CE) marked tests, meeting the performance and legal requirements for their use in Europe, should be utilised for HIV testing. Although infection by HIV-2 and HIV-1 group O and N strains is very rare in Italy, commercial kits that can detect the presence of both HIV-2 and group N and O HIV-1 antigens and antibodies should be used, since these viruses can circulate in Italy, as a consequence of possible immigration of individuals coming from geographical areas where these viruses are present. In addition, in order to keep abreast of kit technology and to strengthen control of the quality of kits used for the diagnosis of HIV infection, it is recommended to create a

Kit Evaluation Group (KEG) committee along the lines of those already present in different European countries [15].

Even though tests comply with European criteria for minimal manufacturing standards, the performance of each test may vary among laboratories. For these reasons, every laboratory should comply with the International Organisation for Standardisation (ISO) rules, at the Comité Européen de Normalisation (CEN) and the Italian Ente Nazionale Italiano di Unificazione (UNI) levels.

Laboratories that perform tests for HIV infection in Italy should have full accreditation status, for both screening and confirmation (or additional testing to confirm screening result). It must be noticed that if a laboratory is accredited only for performing screening tests, but not confirmatory or additional testing, it should not communicate to the patient a positive result obtained only in the screening testing, since this must be further confirmed on (see later). In this case, it should be mandatory for the laboratory to send the sample to a referral laboratory that can perform confirmatory or additional testing. However, laboratories accredited for only screening testing can still deliver a negative result to the patient, since no confirmatory testing is required, unless there are reasons for suspecting a seroconversion, or should the patient report behaviours at risk for HIV infection, or have clinical symptoms of acute infection. In these last cases, a final answer should be provided by only a laboratory accredited for performing confirmatory or additional assays, following the suggested algorithm for the laboratory diagnosis of HIV infection (see later and *Figures 1 and 2*).

HIV TESTING ALGORITHM FOR LABORATORY DIAGNOSIS OF HIV INFECTION IN ITALY

In Italy, there are not shared consensus guidelines for the laboratory diagnosis of HIV infection. Algorithms, which include screening and confirmatory tests, have been developed at a local level, thus often taking into account only local needs [9, 10]. Consequently, many of these algorithms do not always fit at a national level. In addition, confusion among the individuals undergoing voluntary HIV testing can be generated, since, often, there is not a consensus on the significance of a positive or negative result and on the follow up testing time required to provide a final answer, among the different laboratories. Thus, a general, national consensus procedure for HIV laboratory testing is strongly needed in Italy in order to avoid delivery of false results, misunderstanding and frustration in the HIV tested individual and provide uniform and shared criteria for HIV infection diagnosis at a national level.

To this aim we suggest two approaches for laboratory diagnosis of HIV infection to individuals who require HIV testing. Both approaches provide highly reliable results and are suitable for a low HIV prevalence country, as Italy [8]. These approaches could be of help, and taken as models, when drawing guidelines for laboratory diagnosis of HIV infection in Italy.

As a common procedure for both algorithms, the laboratory diagnosis of HIV infection should be performed according to the existing Italian regulations on informed consent to the subject asking for HIV testing and on anonymity. Informed consent should be offered in a context of a pre-test counselling and delivery of the test result should be provided during a post-test counselling (reviewed in [16]). In addition, during both the pre- and the post-counselling, the counsellor should be familiar with basic laboratory terminology, such as sensitivity, specificity and positive and negative predictive values of the tests, to give unambiguous answers to the patient. In particular, the operator should be aware that in Italy, as well as in most countries in Western Europe, low HIV prevalence may lead to false positive results in the screening tests, whereas false negative results are extremely rare.

During the pre-test counselling, it should be investigated whether the subject requiring HIV testing has had recent behaviours at risk for HIV infection [8]. If the individual reports having had an at risk behaviour less than one week from the test execution, the care provider should keep in mind, and inform the individual asking for HIV testing, that an acute HIV infection can be present, also in case of a negative test result, since both HIV antigen and HIV antibodies can be not present in the serum during the very early acute infection.

Common to both algorithms should be the use of the now commercially available, more sensitive, fourth generation EIA, as screening testing. Fourth generation EIA are able to detect both HIV-specific antibodies and the presence of the viral p24 antigen in blood, as early as 13-15 days from infection, thus notably reducing the time of appearance of HIV markers, in comparison to the third generation tests, which take about 22 days to detect HIV antibodies [11, 17-19].

First suggested algorithm

In *Figure 1*, a first algorithm is proposed that takes into account the use of fourth generation EIA as screening, and WB or LIA as confirmatory testing. The basic principle of the algorithm is the fact that, given the low HIV prevalence in Italy, a negative result of screening test excludes current HIV infection, provided the patient is not in the window period. The significance of a negative result must be clarified in a post-test counselling. In particular, if the patient reports no recent at risk exposure, a negative report for HIV infection can be delivered. However, if the subject refers of a recent possible exposure to HIV, or he/she has declared signs or symptoms that can be compatible with HIV infection, the possibility of an HIV acute infection cannot be discarded.
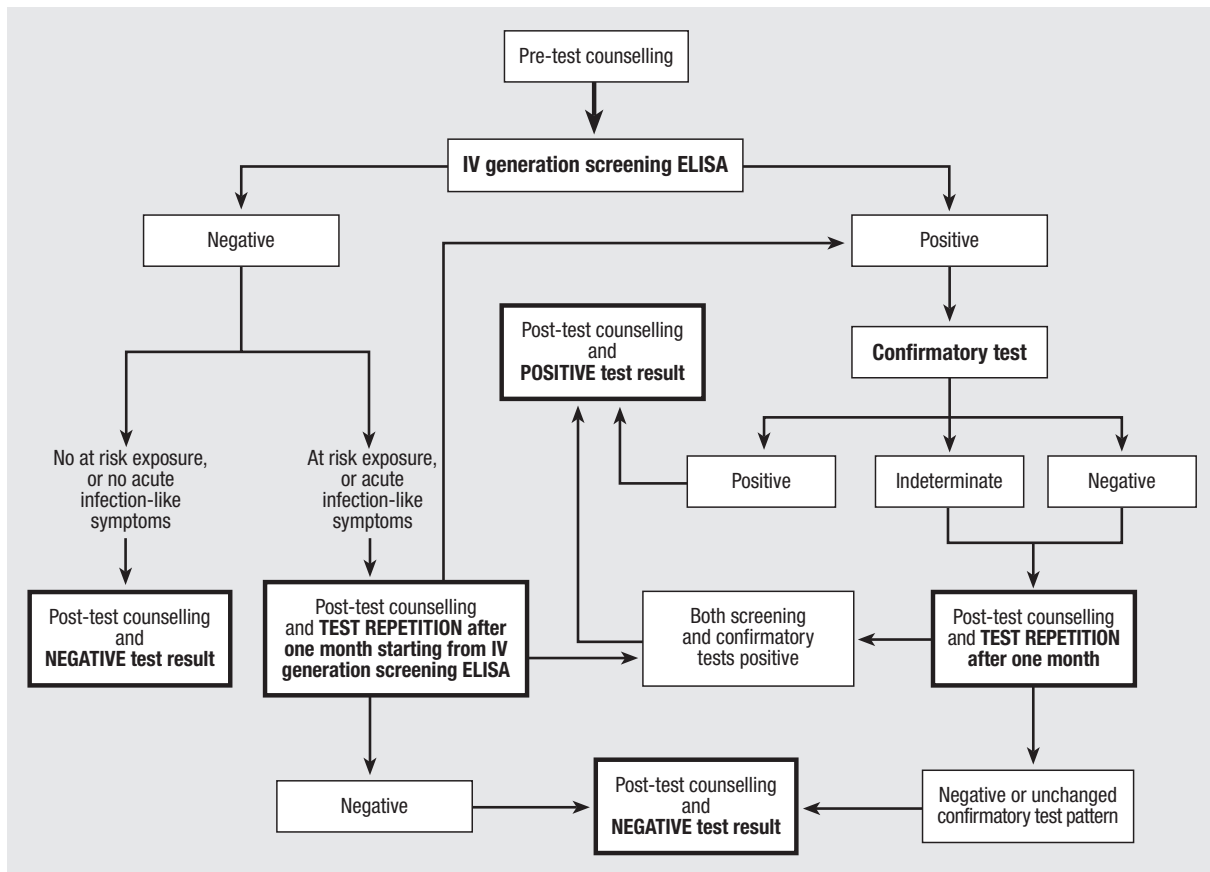
Fig. 1 | *Proposed WB or LIA-based algorithm for the laboratory diagnosis of HIV infection in a low HIV prevalence country.*

In this case, it is advisable to inform the subject that a new test should be made at least 4 weeks after the last reported at risk exposure.

A positive result should, instead, always be confirmed by confirmatory tests, such as WB or LIA. The confirmatory test should be performed from the original test tube, however, in case of haemolysis, or presence of debris, an aliquot should be used, after centrifugation of plasma/serum to clear it of any debris, which can interfere with test result. Concordant positive results between screening test and the confirmatory assay (either WB or LIA) indicate presence of HIV infection. Therefore, in this case, a positive result for HIV infection can be provided to the patient in the context of a post-counselling meeting (reviewed in [16]).

If the WB or LIA result is either negative or indeterminate (reviewed in [11]) additional testing should be required, because the patient can be in the early phases of HIV infection. Therefore, a new testing procedure on a new sample, starting from the screening test and continuing with the confirmatory tests, should be performed at least one month after the reported exposure to HIV. This test can provide different results: if the screening test is either positive or negative, but the WB or LIA pattern is either unchanged from the first testing, or bands number and intensity are reduced, the possibility of a non-specific reaction to HIV antigens is high. The patient should be reassured that the probability of being infected is low, although the picture does not define a final answer, yet. Therefore, a new, final testing for confirmation of absence of HIV infection should be performed after another month. In this regard, it must be said that, with confirmatory tests such as WB, it not so uncommon to see faint bands that, often, are not evident in a subsequent test with a new sample. Often these samples are tested positive with screening tests, although with values very close to cut-off. As said, cases like this must always follow the above described algorithm, but care must be taken not to alarm the tested individual in the presence of such indeterminate results.

In case reactivity in the EIA is increasing and if there are more HIV bands in the WB or LIA (or some bands appear in the case of previous negative confirmatory test), the possibility of a seroconversion is high. This is confirmed if the WB or LIA have reached the criteria for positivity, otherwise the patient should be tested again after two months from the reported exposure.

In the algorithm described in *Figure 1*, it is possi-

ble that the pattern of indeterminate reactivity in the confirmatory tests remains unchanged several times. In that case, a response of non-specific reactivity to HIV antigens and, therefore, of absence of HIV infection, can be finally delivered after 2 months from the reported exposure, if there have been no subsequent exposures to HIV. Rare cases of prolonged or no seroconversion for up to one year have been previously reported [20-22]. However, these studies were all performed with third generation tests and it is highly probable, that if these samples were tested by fourth generation assays, HIV infection could be more easily recognised. In addition, it is also described in literature that HIV antibodies can disappear in patients in advanced stage of disease [23, 24]. These cases should be now all detected by fourth generation tests.

Therefore, according to the described algorithm, a period of one month should be sufficient to define most of the HIV infections. In some cases the testing period can last up to two months after the reported exposure. However, recalling a patient for a follow-up at 6 months can be considered if the tested subject has an impaired ability to develop antibodies, or there is a proven simultaneous infection with another pathogen, such as CMV or HCV, since simultaneous infections with these viruses can delay HIV seroconversion time [25].

If a patient presents clinical symptoms suggestive of HIV infection, but is repeatedly tested negative in the total two months of follow-up from the presumptive behaviour at risk for HIV infection, the rare possibility that he/she is infected with a peculiar HIV strain, not detected by commercially available kits, must be taken into consideration. In this case, additional testing in a specialist laboratory should be considered.

The algorithm depicted in *Figure 1* is designed to reduce the chances of delivering a false positive result. In particular, the purpose of introducing WB or LIA is to achieve the objective to rule out false positive results. However, this strategy suffers of the relatively high frequency of indeterminate results in the confirmatory tests, in particular WB, in countries at low prevalence of HIV infection [26]. These indeterminate results can hinder clinical decisions, create more work, be cost effective and cause inappropriate stress and anxiety to the tested individuals. In some cases, the frequency of indeterminate results in a population with low prevalence of HIV infection, such as blood donors in the United States, can be as high as 45.4% [27]. Numerous articles have been reported, which describe that the frequency of indeterminate WB result can vary from 8% to 45.4%, considering different cohorts of individuals and specimens [27-33] and according to different interpretation criteria. These rates of indeterminate WB were based on the use of algorithms that utilised third generation tests as screening. By using fourth generation-based assays during the screening it is expected that the frequency of indeterminate WB

is even higher [26]. Considering the use of a highly sensitive (100% sensitivity) test during screening and an average frequency of indeterminate WB results of 22%, the cost of the above described algorithm is around 4 €/tested individual. This cost just refers to direct costs and does not include costs for purchasing instrumentation and personnel costs, as well as costs for maintenance.

Second suggested algorithm

In *Figure 2*, an alternative algorithm for the diagnosis of HIV infection in Italy is reported. In this algorithm, confirmation tests (WB and LIA) are substituted by cheaper third generation EIA performed in parallel. In addition, to speed up the testing time in case of follow-up, viral RNA testing is foreseen. Going into the details of the figure, as in the previous algorithm a negative result in the screening test provides a diagnosis of lack of HIV infection, if the individual does not report behaviours at risk for HIV infection. In case the subject reports possible HIV exposure, an aliquot of plasma should be further tested for HIV RNA. If it is tested positive, then it means that the patient is in the serological window period and a diagnosis of HIV infection can be delivered to the patient. The patient must be followed-up in time to confirm development of HIV-specific antibodies.

If the individual is tested positive at the fourth generation screening test, additional testing with two third generation EIA, if possible based on a different detection principle, done in parallel, should be performed. If both the additional EIA are positive, then a diagnosis of HIV infection can be delivered to the patient. If reactivity in the screening test is not confirmed in both the third generation EIA, or it is confirmed in just one of these, the presence of RNA in plasma (no serum) should be investigated on the same specimen. If RNA is tested positive, a diagnosis of HIV infection can be delivered. If RNA test is negative, a follow-up of the patient is required, since the possibility of a seroconversion, although rare, cannot be excluded. For this reason, the patient should be asked to come back for another complete analysis on a new sample after one month from the reported exposure (or one month later if no exposure is reported or if presumptive time of exposure cannot be determined). Sample testing will follow again the algorithm. If at the RNA testing the sample is again negative, a response of lack of HIV infection should be delivered to the patient. If RNA is tested positive, the response of presence of HIV infection should be communicated to the HIV tested individual, and a follow up to verify development of HIV-specific antibodies must be organised.

In very rare occasions, the screening EIA testing can revert negative. In this case, if RNA test continues to be negative, a response of lack of infection can be provided. If RNA test turns to be positive, the patient can be seroconverting and he/she should be followed up after 15-30 days. RNA test turns to be. It must be

said that the last two described scenarios are anecdotic, since in most cases they can be explainable by some mistake of the operator during either one of the two testing rounds. Mistakes can be an exchange of tubes, cross-contamination, erroneous reporting of the result etc.

The above described algorithm is highly sensitive and specific. However, being both the screening and the confirmation based on EIA kits that have 100% sensitivity but a specificity close but not equal to 100%, the possibility to provide a false positive result can exist. We have calculated the probability to provide a false negative result in a population with 0.4% HIV prevalence, like Italy. Considering a scenario with a first screening EIA with 100% sensitivity and 99.6% specificity and the two confirmatory EIA with 100% sensitivity, for both, and 99.9% and 99.5% for the 1st and the 2nd ones, respectively, the probability of providing a false positive result, in a population with a low HIV prevalence, should be less than 0.8/100 000 tested individuals. Direct costs of this algorithm are about 3.6 €/tested individual and do not include costs for personnel, equipment and maintenance.

CONCLUSION

In Italy there are not consensus guidelines for the diagnosis of HIV infection in individuals who wish to know their HIV serostatus, which can drive diagnostic laboratories. At the present, each laboratory/hospital is generating its own standard operating procedures, including diagnostic algorithms, but these are based on the needs and necessities of a limited environment. It is, therefore, advisable, to our opinion, that consensus guidelines for the diagnosis of HIV infection in Italy for individuals who decide to undergo HIV testing are generated and shared among all laboratories and health facilities and Institutions in the country. This will help in avoiding misunderstandings during diagnosis procedure that can create anxiety in the tested individuals, who, due to different testing procedures now in place in Italy, can have different answers depending on the laboratory where HIV testing is performed.

In this paper we have suggested two possible algorithms: one which includes the confirmation by WB or LIA and no RNA testing, and the other one which excludes WB or LIA and includes the execution of two supplemental third generation EIA followed, in case of discordant results, by viral RNA
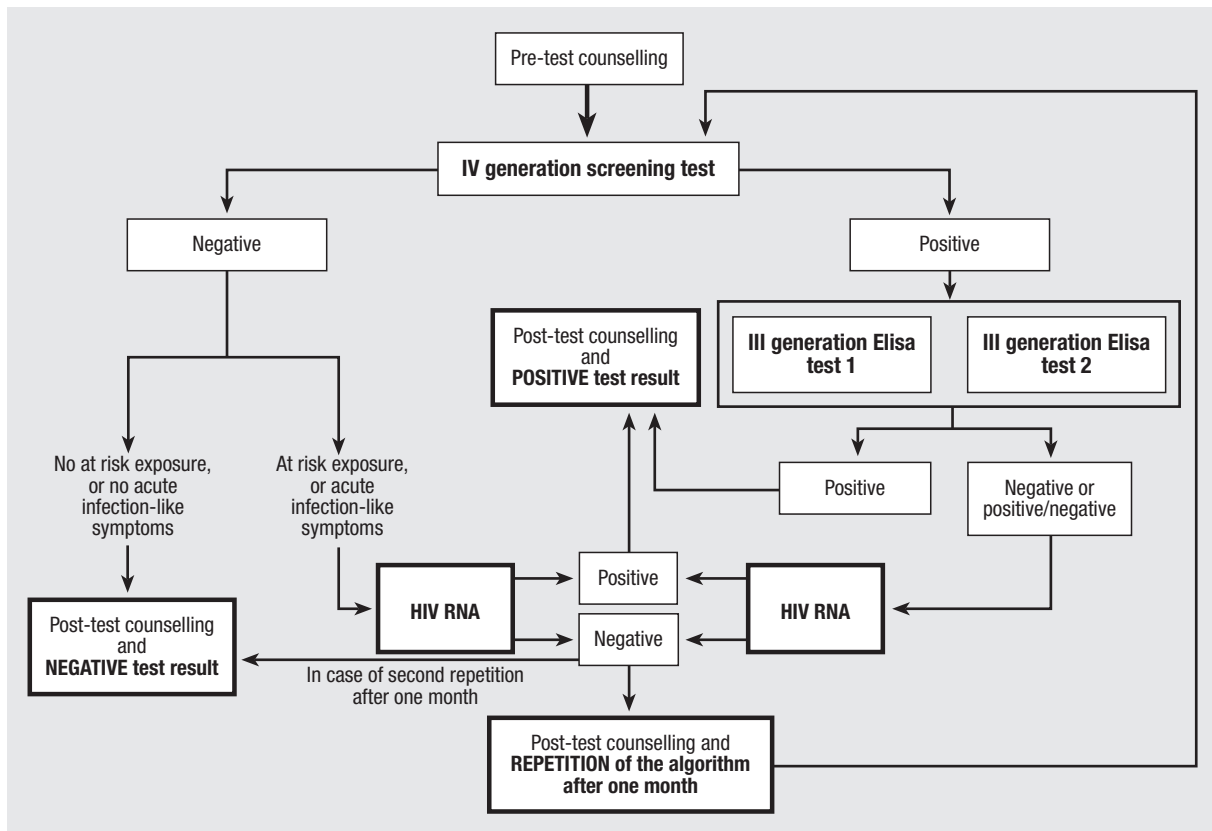

Fig. 2 | *Proposed RNA-based algorithm for the laboratory diagnosis of HIV infection in a low HIV prevalence country.*

testing. The first algorithm can show some limits that have to be considered. One limit is that interpretation of confirmatory assays (in particular WB) is, with rare exceptions, subjective, since the presence and the intensity of HIV-specific bands have to be determined by the operator without the help of an instrument. In addition, these tests can provide indeterminate results with a high frequency, due to either false antibody reactivity to HIV-specific proteins, or reactivity to non HIV proteins (in case of the WB) [26-33]. These indeterminate results can create confusion and stress to the tested individual, and force the operator to delay response of one or two months, in order to conclude the entire diagnostic procedure, because there is the need to wait for the complete HIV-antibody development.

The second suggested algorithm does not include WB and LIA as confirmatory tests, but two third generation EIA performed in parallel after a positive screening test, followed, in case of discordant results, by RNA testing. This algorithm is efficacious in countries with low prevalence and incidence of HIV infection, like Italy, and provides a faster response to the patient, since it assures detection of HIV RNA in the very early phases of the disease (up to two weeks). In case of a chronic disease stage, where the RNA titre can be low, the success of this algorithm is assured by the possibility, through the third generation EIA, to detect antibodies with specificity and sensitivity close to the ones for WB or LIA. This will result in a faster response to the tested individual, thus significantly reducing anxiety and doubts that can be generated in the patient. Furthermore, the use of third generation assays after the screening avoids the indeterminate results, present in WB or LIA, that are often difficult to explain to the tested individual. In addition, this algorithm avoids the need of subjective interpretation of confirmatory tests results, since the results from third generation EIA are numbers referring to optical densities that can only be greater or lower than the cut-off values (*i.e.* positive or negative). Furthermore, last generation RNA assays have standardised and automated procedures and internal controls that should limit errors and misinterpretations. Finally, the presence of the third generation assays and the exclusion of WB reduce the direct costs of this diagnostic strategy.

However, being the algorithm based on EIA in both screening and confirmatory stages, which cannot reach 100% specificity (although some kits declare a specificity very close to 100%), a very small probability (as low as less than 0.8/100 000 tested patients) to deliver false positive results, still exists.

In conclusion, whatever the algorithm, we assert that the development of a consensus strategy for the laboratory diagnosis of HIV infection in Italy is now urgently needed. In addition, we also suggest that a committee of experts would periodically update testing procedures, as technology for diagnosis of HIV infection advances. These suggested procedures can be both of help to the individuals requiring HIV testing to reduce time for diagnosis, and of convenience to the Public Health because a structured, defined and standardized approach can result in a higher efficiency and reduced costs.

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