Fluorescence differentiation supports malaria diagnostics

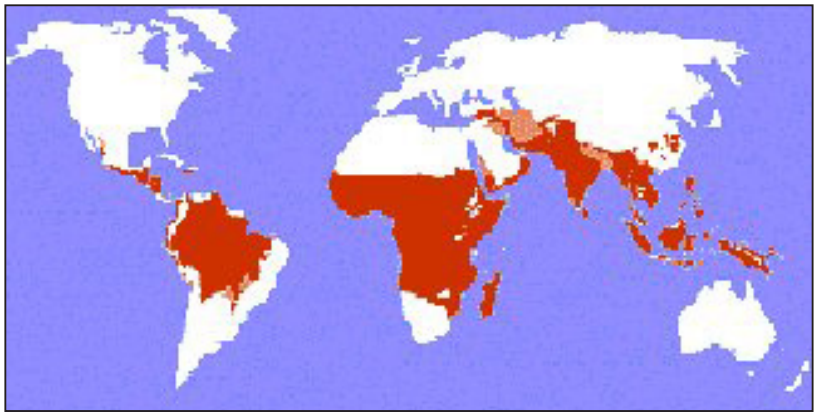

Fig. 1: Geographic distribution of malaria world-wide [4]

Although malaria infections are primarily a problem in tropical countries (Fig. 1), they occur ever more frequently in our part of the world due to tourism and globalization [1]. According to the World Health Organisation (WHO), the number of malaria infections in Europe in the year 2000 had increased tenfold to 15,000 since 1970. Worldwide, 300 to 500 million humans are infected annually [2,3]. Whereas, however, diagnostics and treatment are commonplace in those countries where the disease is endemic, this is unfortunately not the case in Europe.

Frequently, the treating physicians have difficulties giving advice regarding prophylactic measures or even with a fast and clear diagnosis in the event of an infection. Unfamiliar with the clinical picture, they might misinterpret primary clinical symptoms such as fever and chills, headaches, muscle pains, enlarged liver or pancreas, nausea and vomiting, abdominal pain and/or diarrhoea as an influenza-type infection or some similar minor illnesses. This will be especially critical if the patient to be treated had not been in any of the endemic infection regions and thus a malaria infection is not indicated – admittedly a rare case, but it definitely happens. Valuable time will thus pass before the proper diagnosis is reached and treatment can begin. Depending on the type of pathogen, such a delay may be fatal for the patient, in particular due to the fact that European Caucasians have a clearly lower immune defense against this type of infection and the progression of the disease carries a higher risk for the patient [5].

Thus, for the patient as well as for the treating physician and the laboratory, it is of utmost importance to use any available information to enable fast diagnosis and treatment. This is also particularly true for Europe because, here, there is no quick malaria test as part of the standard program in the initial diagnosis of patients with flue-type symptoms; instead, such a test will only be specifically requested if such an infection is suspected. In addition to the clinical symptoms and information about a patient's travels, deviations in the blood count can also be taken into account under certain conditions to request a malaria test or blood film ("thick smear"). The aforementioned will require good knowledge of the potentials and also of the limitations of hematology analysers, as well as the patterns to be expected in the blood count, especially in the early stage of the infection.

In this respect, the sensitivity of the 'thick smear' reference test is to be taken into account which the WHO indicates as being 1:100,000 (for the average trained examiner).

Such sensitivity is also required of the antigen tests but is not always reached; and it is outside the range of any hematology analyser available on the market today. Accordingly, for today's hematology analysers, a corresponding warning message is unfortunately not yet realizable which would indicate a malaria infection with the sensitivity of the current reference method.

Additionally, the situation is even more complex due to the occurrence of different malaria pathogens whose life cycles and occurrence in the host's erythrocytes can certainly be quite different. The following will describe in more detail the two major types of malaria pathogens: *Plasmodium falciparum* and *Plasmodium vivax*. These two types of pathogens are the most frequently occurring and have very characteristic differences, not only in the course of the disease but also in the blood smear and thus in the results of the hematology analyzer as well.

Plasmodium falciparum – Pathogen of malaria tropica

This pathogen is by far the most dangerous of all Plasmodium species since it causes the highest number of deaths – if untreated, approx. 30 % of all infections with this parasite are deadly [6]. At the same time, it is the most frequently occurring pathogen and – with the exception of Europe and Australia – it is endemic on all continents.

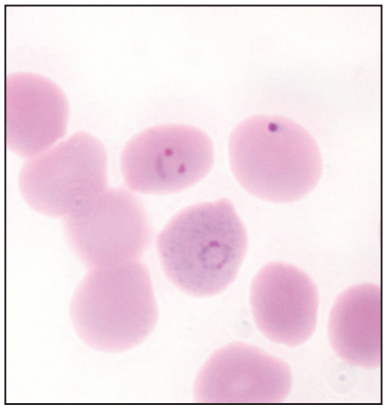

Fig. 2: Trophozoites or ring forms with a *Plasmodium falciparum* infection

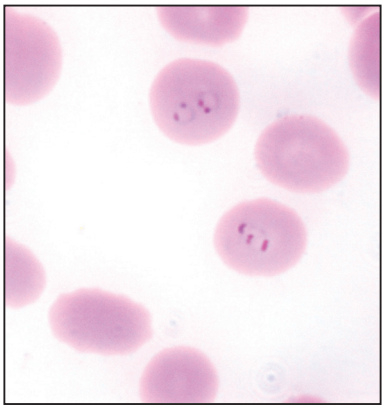

Fig. 3: Double ring forms of the *Plasmodium falciparum* parasite in erythrocytes

After a person is bitten by an infected female Anopheles mosquito – the intermediate host for all malaria pathogens – the pathogen first infests the liver cells. After several development stages, the so-called merozoites go from there into the blood and, in their infectious form, they will penetrate the erythrocytes, not only in the young cells but also in mature cells. There, they will develop to trophozoites which are shown in the blood smear as the typical ring form (Fig. 2 and 3).

Further propagation and development of the pathogens will result in the erythrocytes bursting and – due to the released parasites and their metabolic products – this will finally result in the clinically apparent symptoms such as fever and chills. The usual incubation period is 12 to 14 days, with malaria tropica not having any regular bouts of fever.

Unique for the clinical picture is the change in the surface characteristics of the infested erythrocytes; they quasi 'stick' fast to the endothelial cells of the vessels' inside walls. This adhesive property of the erythrocytes bears the great risk of malaria tropica since vascular occlusions – especially of capillary vessels, e. g. in the brain (the so-called cerebral malaria) or in the kidneys – can result in serious complications, the main cause for the high fatality rate of this type of pathogen. At the same time, this adhesive property also has the effect that only specific forms of affected erythrocytes are detectable in the peripheral bloodstream – mainly the mentioned ring form.

Aside from the fact that the ring form is primarily detectable in the erythrocytes, there are other special features in the blood smear as well:

1. The infested erythrocytes detectable in the blood count are not enlarged.
2. Infested erythrocytes comprise a detectable parasitic nucleic acid part, as opposed to the non-infested red blood cells. In this case, ring forms with two chromatin nodes can occur (see center cell in Fig. 2).
3. Also possible are several ring forms in an erythrocyte (see Fig. 3).
4. Gametocytes only occur after approx. 4 weeks in peripheral blood where they are seldom seen, however. This sexual form will be incorporated by the intermediate host and there develops further until being communicated to yet another human.
5. The percentage of infested erythrocytes can be very high, and a percentage of under 1‰ can already indicate serious illness. This is especially true in view of the fact that the number of pathogens increases greatly while the number of erythrocytes will simultaneously decrease.
6. An additional hematological characteristic is a worsening anemia.
7. Aside from that, thrombocytopenia and leucocytopenia is also typical for the clinical picture, as well as the occurrence of monocytosis. In a very late stage, the number of leucocytes may then increase again.

Fortunately, a malaria tropica infection which was overcome does not bear the risk of the fever recurring months or years after the initial infection since the parasite does not remain in the liver after the first development cycle, as opposed to the other parasitic species causing malaria.

Plasmodium vivax – Pathogen of malaria tertiana

Basically, the life cycle of *Plasmodium vivax* is very similar to that of *Plasmodium falciparum*. However, from its clinical picture, malaria tertiana is not nearly as serious as malaria tropica.

Thus, only young erythrocytes are infested which considerably reduces the total number of infected red blood cells and thus significantly reduces the pathogenicity of the pathogen as compared to *Plasmodium falciparum*. The infected erythrocytes do not adhere to the endothelial cells since they are lacking the 'sticking' properties. Thus, the serious complications of malaria tropica are not present in these cases. Bouts of fever occur frequently, however not always at very regular intervals of approx. 48 hours, with nearly fever-free phases in between [7].

The blood count also shows some differences, as compared with *Plasmodium falciparum*:

1. The hemolysis of young erythrocytes stimulates the production of reticulocytes, with their percentage slightly increasing in the course of the infection. In turn, these young cells are a target for free pathogens in blood.
2. Since erythrocytes do not stick to the endothelial cells of the blood vessels, all maturation forms are found in the red blood cells of the peripheral blood, i. e. aside from trophozoites (ring forms), merozoites, schizonts and gametocytes.
3. *Plasmodium vivax* breaks hemoglobin down to hemozoin; also called Schüffner's dots or malaria pigment and clearly evident as a crystalline structure in the blood smear (see Fig. 4). Since all infested cells continue to circulate in peripheral blood, these cells are also detectable.
4. The infested erythrocytes will increase with the growth and increasing maturity of the pathogens inside the cells before they burst.

However, *Plasmodium vivax* also has a special characteristic which is very unpleasant for the patients concerned: A renewed malaria infection is to be expected approximately every 6 months since some pathogens in the liver remain in a special stage of development (hypnozoit) and will only develop further after reactivation at some later point in time. In extreme cases, these remissions can occur years or even decades after the first infection.

Malaria tertiana will also be caused by *Plasmodium ovale*, whereas the fourth of the malaria pathogens, *Plasmodium malariae*, will cause malaria quartana.

Fluorescence-based technology of XE-2100 and XT-2000i – Where are malaria pathogens found in the scattergram?

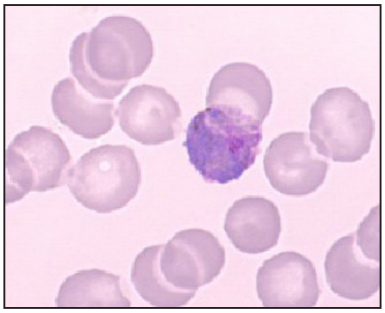

Fig. 4: Schizonts and hemozoins included in an erythrocyte

Nearly all of the hematological characteristics listed under the two malaria pathogens can be detected again in the blood smear as well as in the hematology analysers XE-2100 and XT-2000i. There are primarily two channels of the two SYSMEX XE-2100 and XT-2000i analysers in which the blood parasites are detectable in correspondingly strong infestations:

- RET channel
- DIFF channel

For both channels, special fluorescence dyes are used which very specifically stain nucleic acids. The higher the percentage of nucleic acids (DNA and RNA) in the cell, the greater the resulting fluorescence signal. In the following, both channels are presented in somewhat more detail and information is provided as to where and why erythrocytes infested by malaria pathogens are found in the scattergram. Moreover, there are yet further blood count parameters which, in combination, may additionally indicate a malaria infection and which are the basis for the warning messages indicated by SIS (SYSMEX INFORMATION SYSTEM). Due to a multitude of experiences with individual cases, SYSMEX also offers a corresponding warning message ‘malaria’ in SIS which summarizes the phenomena described in the following in a rule-based system.

RET channel

The RNA percentage of the reticulocytes presents the single morphological differentiation criterion between erythrocytes and reticulocytes, and this also applies for automatic counting (Fig. 5). This nucleic acid percentage is specifically stained by means of a patented fluorescence dye in the RET channel. The higher the nucleic acid percentage of the reticulocytes, the younger the cells and also the greater, at the same time, their fluorescence signal in the RET channel. This is proportionate with the RNA percentage of the reticulocytes, i. e. mature erythrocytes have only a very low or, respectively, no fluorescence signal and are thus found very far left on the X-axis. Leucocytes which are not lysed in this channel have a cell nucleus with significantly more nucleic acids than reticulocytes and are thus outside the scattergram.

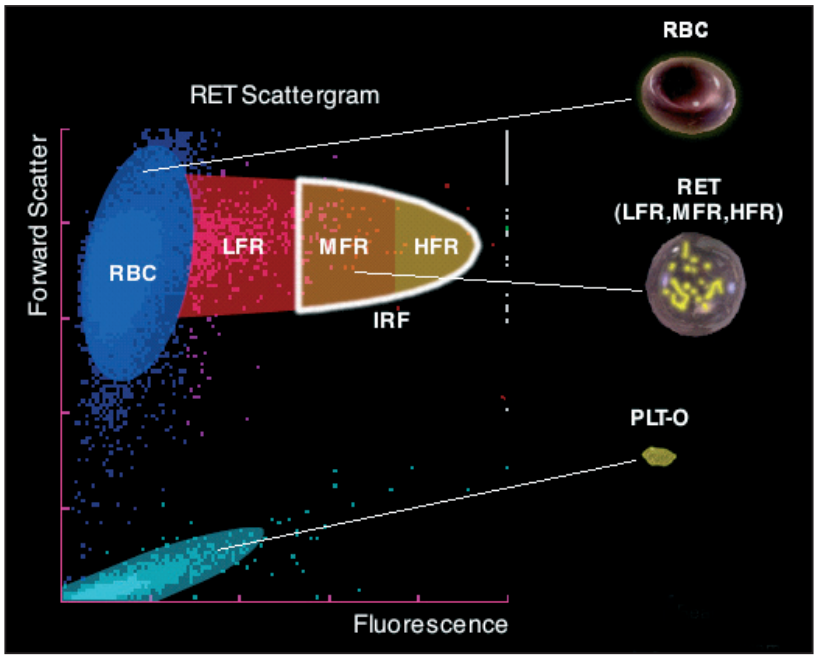

Fig. 5: RET channel of *XE*-2100 and *XT*-2000i. On the X-axis, the fluorescence intensity based on the cell's nucleic acid percentage is ascendingly marked off. The Y-axis, in contrast, ascendingly marks off the size of the cells.

Malaria pathogens also contain nucleic acids. These too are stained and detected in the RET channel. In view of the different hematological characteristics of the two most important malaria pathogens *Plasmodium falciparum* and *Plasmodium vivax*, the appearance of the two types is very different in the scattergram. Thus – due to the low total number of infected erythrocytes and the initially mentioned low sensitivity for the detection of these blood parasites – infestation with *Plasmodium vivax* pathogens cannot be detected, at least not in the RET channel, in the hematology units XE-2100 and XT-2000i.

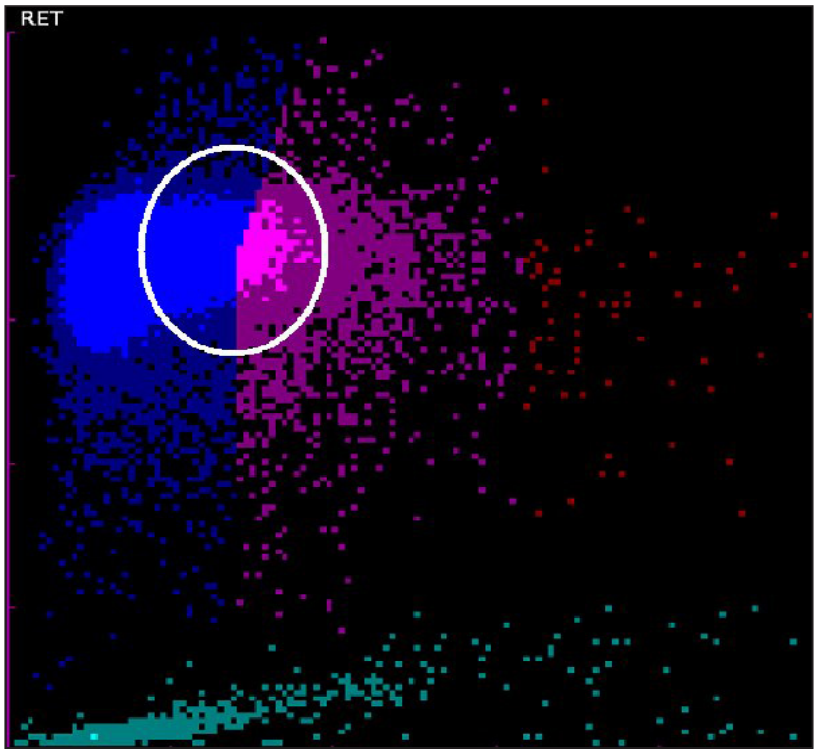

Fig. 6: RET scattergram of a patient infected with *Plasmodium falciparum*

The situation is quite different for a malaria infection with *Plasmodium falciparum*. The percentage of infested erythrocytes is relatively high at times while, at the same time, several ring forms and those with several chromatin nodes can occur. Both will result in a significant increase in the percentage of erythrocytes which have quite a considerable nucleic acid concentration. Fig. 6 shows exactly where the affected erythrocytes are found with malaria tropica: The erythrocytes infested with *Plasmodium falciparum* have a higher fluorescence signal in the RET scattergram due to the parasitic nucleic acids. At the same time, however, the infested cells are not enlarged and, therefore, the forward scatter light is not increased.

Warning messages from the RET channel

All haematology analysers currently available on the market are designed to determine anomalies in the blood count, not, however, to detect specific components of blood cells. The sensitivity of reliably diagnosing a malaria infection is too low – for example in the form of a possible warning message such as 'malaria' by the haematology analyser. Thus, the 'thick smear' test still remains indispensable. This also applies for the XE-2100 and XT-2000i analysers which detect very specifically the intracellular nucleic acid content of blood cells but are unable to distinguish between parasitic nucleic acids and cell-specific DNA/RNA or RNA with reticulocytes.

For malaria infections by *Plasmodium falciparum* which are also visible in the RET scattergram, the XE-2100 and XT-2000i analysers usually show the following results:

- 'RET Abn Scattergram' is the indicated warning message
- Apparently greatly increased 'reticulocyte count', especially of the more mature 'reticulocytes'. Particularly the LFR value (Low Fluorescence Ratio) of the reticulocyte parameters can be significantly increased and reach a percentage of approx. 90 %.
- The number of very young reticulocytes – IRF (Immature Reticulocyte Fraction) – is low or, normal while at the same time the 'reticulocyte count' is apparently greatly increased. Physiologically, such a greatly increased total number of reticulocytes is not possible with normal production. However, the constellation of these parameters may be triggered by an infection with *Plasmodium falciparum*.

In particular the number of reticulocytes and the percentage value of LFR could be used to examine whether the therapy is successful in case of a malaria infection. The number of reticulocytes provided by the analyser should decrease significantly if the parasite infestation in erythrocytes is declining.

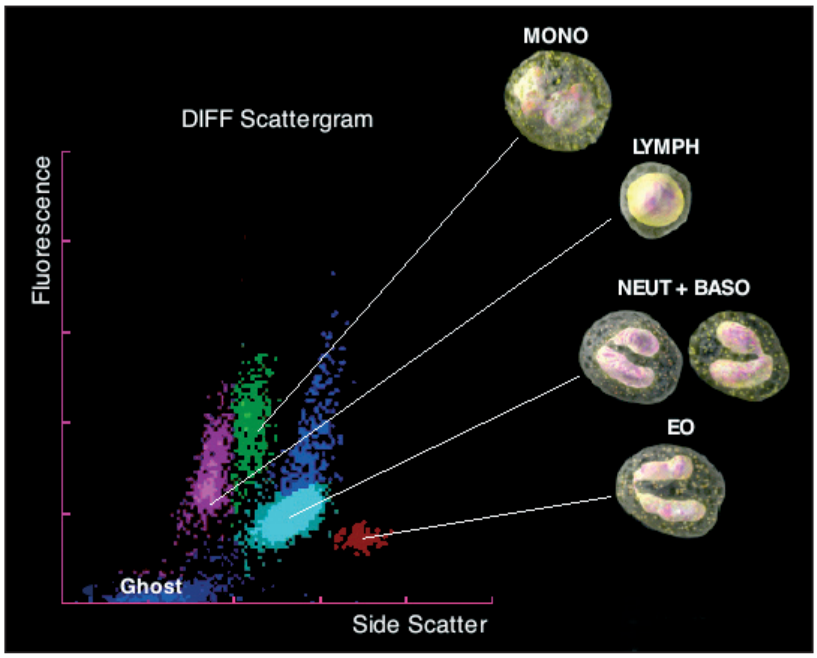

Fig. 7: DIFF channel of *XE*-2100 and *XT*-2000i. On the Y-axis, the fluorescence intensity based on the cell's nucleic acid percentage is ascendingly marked off. In contrast, the X-axis ascendingly marks the increasing complexity of the cells, particularly based on the percentage of granules

DIFF channel

The DIFF channel differentiates leucocytes according to their nucleic acid content and their internal structure, not according to their size (Fig. 7). This ensures an exact count of lymphocytes, monocytes, eosinophils and neutrophils. At the same time, it offers excellent registration of immature blood cells and thus reliable flagging of even highly pathological samples.

Reason for this is the considerable nucleic acid percentage of young actively dividing blood cells as compared with matured leucocytes in blood circulation, because the more immature the blood cell, the higher its RNA percentage.

Even if the erythrocytes infested with malaria parasites have a significantly increased nucleic acid percentage versus non-infested erythrocytes, the resulting signal will still be much smaller than from any nucleated cell which contains DNA and thus always results in a stronger fluorescence signal. Accordingly, erythrocytes or reticulocytes, infested or not, will be found in the DIFF scattergram in the 'ghost area' (darkblue area in Fig. 7).

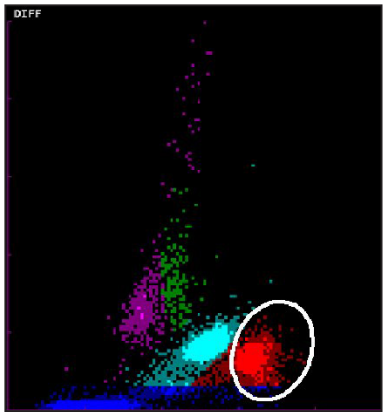

Fig. 8: Schizonts and gametozonts cause the clearly increased side scatter light signal, especially in the detection area of the eosinophils.

Especially in case of *Plasmodium vivax* infections, increased side scatter and fluorescence scatter light can be observed in the detection area of the eosinophils – in the presence of those free pathogen forms of schizonts and gametozonts in the DIFF scattergram (Fig. 8). Even the released hemozoin might contribute to this increased side and fluorescence signal. In contrast, infections with *Plasmodium falciparum* are less specifically detected in the DIFF scattergram.

Warning messages from the DIFF channel

With malaria infections by *Plasmodium vivax* which are also visible in the DIFF scattergram, the XE-2100 and XT-2000i analysers usually show the following results:

- As the results in Fig. 8 show, the eosinophil result will be completely blocked due to the interfering additionally detected particles.
- Frequently, 'Atypical Lymph?' is also found, caused by reactive lymphocytes.

SIS rules for malaria warning messages

If there is a heavy infestation with malaria pathogens which clearly shows in the RET or DIFF scattergram and results in different analyser warning messages, the result can be reliably interpreted according to technical aspects by means of pertinent rules in the SYSMEX INFORMATION SYSTEM (SIS). Such technical validation will then result in a more precise warning message: 'Malaria? Check smear!'. Prerequisite for such a warning message by SIS will be that the above described malaria-typical characteristics are combined with basic haematology rules for RET and DIFF scattergrams. At the same time, the original analyser warning messages will no longer be shown.

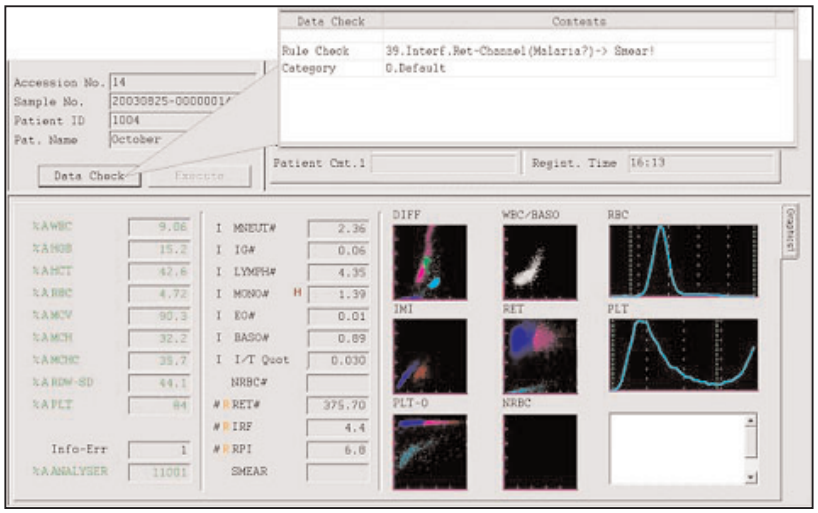

Fig. 9: Finding of a *Plasmodium falciparum* infection which generated the warning message 'Malaria?' by means of *SIS*.

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