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MOLECULAR TOOLS FOR THE RAPID DETECTION OF TRYPANOCIDAL DRUG RESISTANCE IN ANIMAL TRYPANOSOMES.

OUTILS MOLECULAIRES POUR LA DETECTION RAPIDE DE LA RESISTANCE AUX TRAITEMENTS TRYPANOCIDES DES TRYPANOSOMIASES ANIMALES

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Résumé

A l'heure actuelle, des cas de résistance aux traitements trypanocides (RTT) ont été signalés dans 16 pays d'Afrique, mais des enquêtes à grande échelle n'ont été réalisées que dans 9 de pays. Peu d'information sont actuellement disponibles sur la prévalence et la répartition de la RTT et ceci est dû au fait que les outils de diagnostic actuels sont onéreux, prennent du temps et requiert une main d'œuvre importante. De nouvelles méthodes de diagnostic ont été mises au point grâce à une meilleure connaissance des mécanismes de résistance à l'isometamidium (ISM) et au diminazène (DA). Un test *Mbo*II-PCR-RFLP a été mis au point pour le diagnostic de la résistance à l'ISM chez *Trypanosoma congolense*. Ce test est basé sur la présence d'une mutation (une insertion d'un codon GAA) présente dans certains des phénotypes résistants. La prévalence réelle de résistance à l'ISM se trouve sous-estimée à cause de l'existence de mécanismes alternatifs de résistance à l'ISM qui ne sont pas mis en évidence par cette technique. Le diagnostic par des méthodes moléculaires de la résistance à l'ISM se révèle plus compliqué que celui de la résistance au DA en raison d'un mode de transport de la molécule à travers des membranes biologiques, qui semble beaucoup moins spécifique que le transport du DA par les transporteurs puriques de type P2. Un test *Bcl*I-PCR-RFLP a été mis au point pour la détection de la résistance au DA chez *T.congolense* et *T. brucei*. Ce test est basé sur le remplacement d'un codon GTC par un codon ATC chez les souches résistantes. Ce nouvel outil moléculaire semble surestimer la prévalence de la résistance au DA quand on le compare aux résultats des tests effectués sur des souris à une dose de 20mg/kg, mais semble être un bon indicateur de l'apparition ou du développement de phénomènes de résistance dans une région donnée. Des données sur la validation de ces deux outils PCR-RFLP seront présentées lors de cette intervention.

Summary

Currently, there are 16 African countries in which trypanocidal drug resistance has been reported and there are only 9 of those countries in which large scale surveys

were carried out. In many countries we lack baseline information about the prevalence of drug resistance (DR) in trypanosomes which is mainly due to the fact that the currently available tools for the detection of DR are very laborious, expensive and time consuming. Based on the knowledge of the mechanisms involved in resistance to isometamidium (ISM) and diminazene (DA) new molecular tools of diagnosis have been developed which allow a much faster diagnosis of DR than the conventional tests. A *Mbo*II-PCR-RFLP test was developed for the detection of ISM resistance in *Trypanosoma congolense.* It is based on the detection of a mutation (GAA codon insertion) present in some of the resistant phenotypes. Because other mechanisms of resistance to ISM exist, which are not detected by this technique, this tool is underestimating the real prevalence of ISM resistance. The molecular diagnosis of ISM resistance appears to be quite complicated because the transport of this hybrid molecule through biological membranes appears to be far less specific than the transport of DA by P2-type purine transporters. A *Bcl*I-PCR-RFLP test was developed for the detection of DA resistance in *T. congolense* and *T. brucei* which is based on the detection of a GTC-ATC codon shift between the sensitive and resistant strains. This new molecular tool seems to overestimate the prevalence of DR to DA when compared to the tests in mice at a dose of 20mg/kg, but it appears to be a good indicator of the development of resistance in a particular area. Data on the validation of both PCR-RFLP tests for the detection of trypanocidal drug resistance will be presented.

PCR-RFLP for the detection of ISM resistance

Molecular methods for the diagnosis of ISM resistance were recently developed by Delespaux *et al.* and Afework *et al.* (Delespaux *et al.* 2005; Afework *et al*. 2006). The first method (Delespaux *et al.* 2005) allows the discrimination between ISM-sensitive and resistant strains of *T. congolense* by *Mbo*II-PCR-RFLP. This test is based on the polymorphism observed in a 381 bp fragment (sensitive strains) or 384 bp fragment (resistant strains) of a putative gene presenting some homologies with an ABC transporter. The second method (Afework *et al*. 2006) has been developed to distinguish ISM-resistant from sensitive strains of *T. brucei*. This *Sfa*NI-PCR-RFLP test is based on the polymorphism of a 677 bp fragment of the TbAT1 gene. It is postulated by the author that the same set of six point mutations is conferring resistance to the melarsenoxyde cysteamine cymelarsan (an arsenical diamidine) and to ISM (diamidine compound) and that the detection of one of those six mutations allows for the reliable diagnosis of sensitivity or resistance to ISM.

Correlation with in vivo tests

The reference test for the determination of drug resistance remains the standardized single dose mouse test (Eisler *et al.* 2001). The correlation of the *Mbo*II-PCR-RFLP with the mouse test was reported to be 85.7% for *T. congolense* isolates (n=30) originating from different areas throughout the tsetse fly-belt (Delespaux *et al.* 2005). However, the same tool used on 20 *T. congolense* isolates originating from Ethiopia and Burkina Faso and 9 isolates from Zambia showed a correlation of only 60% (Delespaux, unpublished results) and 75% (Dayo 2005), respectively. In a recent survey in Cameroun (Adamaoua Plateau), the *MboII*-PCR-RFLP identified only 4 strains as resistant among 12 isolates confirmed to be resistant in the mouse test (33.3%) (Mamoudou, personal communication). This clearly indicates that there might be ISM-resistant strains that have developed alternative pathways of resistance that are not detectable by the *Mbo*II-PCR-RFLP test. This is not surprising since Ross and Sutherland (1997) had suggested the existence of more than one mechanism of resistance to ISM.

Data on the correlation of the *Sfa* NI-PCR-RFLP test described by Afework *et al*(2006) and in vivo tests are only available for eleven ISM-sensitive strains from Uganda, two ISM-sensitive reference strains from Kenya and two multidrug resistant reference strains from Somalia. The first resistant reference strain CP547 being resistant to ISM, DA, quinapyramine, melarsoprol, homidium and pentamidine, the second resistant reference strain CP2469 being resistant to both ISM and DA but not to quinapyramine (Afework *et al.* 2006). There is clearly a need to further validate the *Sfa*NI-PCR-RFLP test using as resistant reference strains isolates that were characterized as resistant to ISM but sensitive to the other drugs. From the study of Afework *et al*. (2006) it is difficult to confirm whether or not the *Sfa*NI polymorphism is specifically related to ISM resistance or whether the observed restriction patterns are linked to the resistance to the other drugs. However, it should to be noted that field observations suggest that cross resistance between ISM and DA does not exist (Sinyangwe *et al.* 2004). On the other hand, cross resistance has been observed between homidium (which is not a diamidine compound) and ISM (Peregrine *et al.* 1997). From the available data, it appears that none of the recently developed molecular tools for the detection of ISM resistance is fully satisfactory and that further field validation and investigations are required.

PCR-RFLP and Allele-specific PCR (AS-PCR) for the detection of DA resistance

Recently, the analysis of the P2-type purine transporter TcoAT1 of *T. congolense* by means of the SSCP led to a simple *Bcl*I-PCR-RFLP test allowing for the rapid identification of DA resistant stocks (Delespaux *et al.* 2006). This test is based on a single nucleotide permutation (G to A) observed in the DA resistant strains that can be easily detected through *Bcl*I restriction of the amplicon. This single point mutation confers a Val 306 Ile permutation in the purine transporter. Interestingly, it appears that in *T. congolense* a single amino acid permutation is sufficient to induce resistance to DA. In *T. brucei,* a conserved set of six point mutations was described in the TbAT1 gene of melarsoprol resistant strains (Mäser *et al*. 1999). Evidences that DA is exclusively accumulated by the TbAT1 gene was reviewed by Delespaux and De Koning (2007). Although resistance to melarsoprol, and more particularly high levels of resistance could be due to additional factors or mechanisms such as intervention of the High Affinity Pentamidine Transporter (HAPT1) or ABC transporters (Luscher *et al*. 2006; Delespaux & de Koning 2007), the role of the TbAT1 gene remains crucial. *Sfa*-NI polymorphism was used to trace DA resistance in *T. brucei* isolates (Delespaux, unpublished results) together with the AS-PCR described by Nerima *et al*. (2007). This AS-PCR correlates 100% with the *Sfa*-NI PCR-RFLP, is cheaper and quicker than the *Sfa*-NI PCR-RFLP.

Correlation with in vivo tests

The *Bcl*I-PCR-RFLP was validated using 26 *T.congolense* strains (Delespaux *et al*. 2006). A correct diagnosis was obtained with 14 strains which were identified as sensitive at 20mg/kg of DA and with 9 being diagnosed as resistant at 20mg/kg in the standardized mouse test. Three strains, sensitive in the mouse test at the commonly accepted discriminatory dose of 20mg/kg, were identified as resistant by the *Bcl*I-PCR-RFLP. However, these 3 strains relapsed at lower doses of DA (5mg/kg). Some *T. congolense* strains (n=9) isolated in Cameroon on the Adamaoua Plateau were tested both by the *Bcl*I-PCR-RFLP and the mouse test (Delespaux, unpublished results). All nine strains were found resistant in both tests.

The excellent correlation between the *Bcl*I-PCR-RFLP and the standardized mouse test in both studies (32/35 or 91.4%) is encouraging but should be confirmed using

a larger number of strains. The fact that 3 strains which were diagnosed as sensitive in the mouse test relapsed at lower doses than the commonly accepted discriminatory dose of 20 mg/kg suggests a higher sensitivity of the molecular test as compared to the mouse test.

Perspectives

The molecular diagnosis of DA resistance in trypanosomes is facilitated by the specificity of the transport mechanism. Further field validation of the *BclI*-PCR-RFLP and *AS-PCR* for *T. congolense* and *T .brucei* respectively, is necessary but the good correlation between in vivo tests and molecular tools is very likely to be confirmed further. The molecular diagnosis of ISM resistance appears to be more complicated because the transport of this hybrid molecule through biological membranes seems to be less specific than the transport of DA. Several pathways are probably implicated in the process and several diagnostic tests will have to be performed depending on the number of importers or extruders that are potentially involved. ISM-resistance might be caused by a combination of reduced uptake and increased efflux, those two phenomena acting synergistically. The higher the efflux, the higher the energy (ATP) consumption, which in turn provokes a decrease in the mitochondrial potential and a correlated reduced accumulation of ISM in the kinetoplast. It is also very likely that the level of expression of the genes coding for those importers or extruders as well as a gene dosage for each of the transporters will be necessary for a more precise definition of the resistant phenotypes. To achieve this, the quantitative Real-time PCR will be a powerful tool but will still require live trypanosomes with all the practical implications involved such as the fact that it is labour intensive and cost.

Conclusions

Although these newly developed molecular markers still need to be validated further, there are good prospects that they will allow a much faster detection of trypanocidal drug resistance (a few days instead of 2 to 3 months). This will provide the opportunity to carry out much needed area-wide resistance surveys to determine the current prevalence and the spread of resistance genes in trypanosome populations. Furthermore, molecular markers for trypanocidal drug resistance will make it possible to determine, for example, the stability of drug resistance genes after withdrawal of the drug selection pressure or to study the dominance or recessiveness of drug resistance alleles and will contribute substantially to our understanding of the epidemiology of drug resistance. This kind of research will improve the insight in the mechanisms contributing to the development of drug resistance and should ultimately lead to the development of better strategies to delay the development of drug resistance in trypanosomes.

References

Afework,Y., Mäser,P., Etschmann,B., Samson-Himmelstjerna,G., Zessin,K.H. & Clausen,P.H. 2006. Rapid identification of isometamidium-resistant stocks of *Trypanosoma b. brucei* by PCR-RFLP. Parasitol Res **99,** 253-261.

Dayo,G.K. 2005. Corrélation entre le test sur souris et la PCR-RFLP pour la détection de la résistance au chlorure d'isometamidium de souches de trypanosoma congolense de différentes pathogénicités. IMTA, Thèse de MSSAT **34,** 1-37.

Delespaux,V., Chitanga,S., Geysen,D., Goethals,A., Van den Bossche,P. & Geerts,S. 2006. SSCP analysis of the P2 purine transporter TcoAT1 gene of *Trypanosoma congolense* leads to a simple PCR-RFLP test allowing the rapid identification of diminazene resistant stocks. Acta Tropica **100,** 96-102.

Delespaux,V. & de Koning,H.P. 2007. Drugs and drug resistance in African trypanosomiasis. *Drug Resist Update* **10,** 30-50.

Delespaux,V., Geysen,D., Majiwa,P.A.O. & Geerts,S. 2005. Identification of a genetic marker for isometamidium chloride resistance in *Trypanosoma congolense*. Int J Parasitol **35,** 235-243.

Eisler,M.C., Brandt,J., Bauer,B., Clausen,P.H., Delespaux,V., Holmes,P.H., Ilemobade,A., Machila,N., Mbwambo,H., McDermott,J., Mehlitz,D., Murilla,G., Ndung'u,J.M., Peregrine,A.S., Sidibe,I., Sinyangwe,L. & Geerts,S. 2001. Standardised tests in mice and cattle for the detection of drug resistance in tsetse-transmitted trypanosomes of African domestic cattle. Vet Parasitol **97,** 171-182.

Luscher,A., Nerima,B. & Mäser,P. 2006. Combined contribution of TbAT1 and TbMRPA to drug resistance in *Trypanosoma brucei*. Mol Biochem Parasitol **150,** 364-366.

Mäser,P., Sutterlin,C., Kralli,A. & Kaminsky,R. 1999. A nucleoside transporter from *Trypanosoma brucei* involved in drug resistance. *Science* **285,** 242-244.

Nerima,B., Matovu,E., Lubega,G.W. & Enyaru,J.C. 2007. Detection of mutant P2 Adenosine transporter (TbAT1) gene in *T. b. gambiense* isolates from Northwest Uganda using Allele-specific PCR. Trop Med Int Health **in press**.

Peregrine,A.S., Gray,M.A. & Moloo,S.K. 1997. Cross-resistance associated with development of resistance to isometamidium in a clone of *Trypanosoma congolense*. Antimicrobial Agents and Chemotherapy **41,** 1604-1606.

Ross,C.A. & Sutherland,D.V. 1997. *Trypanosomiasis and Leishmaniasis: Biology and Control*.
Hide et al.

Sinyangwe,L., Delespaux,V., Brandt,J., Geerts,S., Mubanga,J., Machila,N., Holmes,P.H. & Eisler,M.C. 2004. Trypanocidal drug resistance in Eastern province of Zambia. *Vet Parasitol* **119,** 125-135.