Molecular techniques: Present and future

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During recent years, molecular techniques in the microbiological laboratory have gained in significance. With respect to the diagnosis of the infection with *Helicobacter pylori (H. pylori)*, modern molecular methods not only allow for the detection of the pathogen but also for clarithromycin susceptibility testing. This is of importance because clarithromycin is an integral part of first line therapies to treat *H. pylori* infection and as demonstrated by a meta-analysis of published data, susceptibility or resistance to clarithromycin results in eradication rates of 81-95% and 0-48%, respectively. Since clarithromycin is a widely used antimicrobial drug, the prevalence of clarithromycin resistant *H. pylori* strains is increasing continuously.

In *H. pylori*, resistance to clarithromycin is mainly due to an adenine-to-guanine transition at positions 2142 and 2143, and to an adenine-to-cytosine transversion at position 2142, which are included in the peptidyltransferase loop of the 23S rRNA. The first technique described for detection of point mutations associated with clarithromycin resistance utilises a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach. Other tests based on the principle of reverse hybridisation with labelled probes, e. g. DNA enzyme immunoassay (DEIA) and PCR line probe assay (LiPA), followed. However, the most convenient techniques in routine laboratory practice are probably fluorescent in situ hybridisation (FISH) and real-time PCR followed by melting curve

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analysis. So far, these tests have been used for the detection of resistance to clarithromycin either in cultured *H. pylori* isolates or in gastric biopsies with the exception of a biprobe based real-time PCR assay, which also allowed for the accurate detection of *H. pylori* and of point mutations associated with clarithromycin resistance in stool specimens.

The biprobe real-time PCR technology uses sequence-specific probes (biprobes) labeled with the fluorophore Cy5. When the probe hybridises to the target sequence (amplicon), Cy5 is excited by the energy transfer from SybrGreen I resulting in an increase of emitted light. In the presence of mismatched bases between the probe and the target, melting curve analysis reveals a lower melting temperature than in the case of a perfectly matched sequence. At present, a commercially available test exists, which was developed on the basis of this technology (*H. pylori* ClariRes assay). This assay has been approved for in vitro diagnostics and currently represents the only non-invasive test allowing for *H. pylori* detection and clarithromycin susceptibility testing.

In a recent study, stool specimens of 214 symptomatic children were collected and examined by both *H. pylori* ClariRes assay and the monoclonal stool antigen test Amplified-IDEIA-HpStAR. In case of discrepancy between the two tests, *H. pylori* status was determined by ^{13}C-UBT and serology. Discrepant results between the two tests were found in nine cases (test agreement: 95.8%). One false negative result was shown by the PCR and two by the stool antigen test. However, six samples were false positive by the latter resulting in 96.6% specificity and a positive predictive value of only 85% due to the low prevalence of the infection (16.8%) in the studied population. This data suggest that the novel *H. pylori* ClariRes assay, also allowing for clarithromycin susceptibility testing, might be the better alternative to other non-invasive tests e.g. stool antigen test in particular in pediatric patients. It is conceivable that in the near future more non-invasive tests also allowing for susceptibility testing to antibiotics other than clarithromycin will be available.

Apart from diagnostic purposes, molecular techniques have been widely used for epidemiological investigations. In fact, although the pathogen has been first cultivated and clearly identified as a potential cause of illness over 20 years ago, the source(s) of infection and the route(s) of transmission have not yet been identified. As no significant non-human or environmental source for this infection has been identified, person to person spread is almost certainly the main mode of transmission. Moreover, since *H. pylori* infection has been shown to be typically acquired in early childhood, a predominantly intrafamilial transmission has been postulated. This is supported by the fact that the infection clusters in families and a child's risk of being infected is supposed to be largely determined by the presence or absence of infected family members. Indeed, using molecular typing methods it could be shown that unrelated individuals harbor distinct *H. pylori* isolates, while clonal lineages have been discerned within families clearly indicating intrafamilial transmission. However, available data are scarce and often based on small samples and may therefore not

allow for further characterization of transmission patterns. This reflects the difficulties in obtaining gastric biopsies from children and asymptomatic relatives. However, endoscopy has been the only possible approach, since molecular typing methods used so far may deliver accurate results only when applied on cultured *H. pylori* isolates.

Recently, a non-invasive protocol was developed allowing not only for the detection but also for typing of *H. pylori* in stool. This protocol is also based on the biprobe technology consisting of two highly *H. pylori*-specific real-time-PCR assays (*ureC* and *recA*) followed by melting curve analysis of the probe-amplicon duplex and - if necessary - subsequent sequence analysis of the amplicons. Target sequences had to meet a number of criteria e. g. to be present in every *H. pylori* strain and to consist of at least one variable region in between conserved regions; mismatches in the variable region should allow for the discrimination between different strains. The length of the amplicons was also of importance and was restricted between 120 and 180 bp.

Analytical validation revealed for both PCR assays low limits of detection and by the combination of both assays a discriminatory capacity of 76.4%. Subsequent sequence analysis of the *ureC* amplicon resulted in a dramatic increase of the discriminatory capacity to 100%. The presence of stool DNA did not show to have any influence on the melting temperature of probe-amplicon duplexes. Thus, a shift in the melting temperature was not observed when different negative stool specimens were spiked with DNA of the same *H. pylori* strain. During clinical validation using stool specimens of unrelated individuals of known *H. pylori* status, the method showed high sensitivity with respect to the detection of the pathogen and excellent discriminatory capacity. In an ongoing study, the examination of stool specimens of closely related individuals showed clonal identities in five out of the six European and in seven out of the eight African households. In two African households, two different clonal lineages were found each; in one case, one individual was infected with both strains.

Since stool specimens are much easier to obtain than gastric biopsies, especially in asymptomatic individuals, this novel method may be suitable for large-scale epidemiological investigations, which should finally allow for the characterization of the intrafamilial transmission patterns of *H. pylori*. This information is of great importance because it may have an impact in terms of reduction of the risk of acquisition of the pathogen.