Comparison of the novel quantitative ARMS assay and an enriched PCR-ASO assay for K-ras mutations with conventional cytology on endobiliary brush cytology from 312 consecutive extrahepatic biliary stenoses

van Heek, N.T.; Clayton, S.J.; Sturm, P.D.J.; Walker, J.; Gouma, D.J.; Noorduyn, L.A.; Offerhaus, G.J.A.; Fox, J.C.

DOI
10.1136/jcp.2004.022707

Publication date
2005

Published in
Journal of clinical pathology

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

Download date:03 Aug 2023
Comparison of the novel quantitative ARMS assay and an enriched PCR–ASO assay for K-ras mutations with conventional cytology on endobiliary brush cytology from 312 consecutive extrahepatic biliary stenoses

N T van Heek, S J Clayton, P D J Sturm, J Walker, D J Gouma, L A Noorduyn, G J A Offerhaus and J C Fox

doi:10.1136/jcp.2004.022707

Updated information and services can be found at:
http://jcp.bmj.com/cgi/content/full/58/12/1315

These include:

References

This article cites 45 articles, 13 of which can be accessed free at:
http://jcp.bmj.com/cgi/content/full/58/12/1315#BIBL

1 online articles that cite this article can be accessed at:
http://jcp.bmj.com/cgi/content/full/58/12/1315#otherarticles

Rapid responses

You can respond to this article at:
http://jcp.bmj.com/cgi/eletter-submit/58/12/1315

Email alerting service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article

Topic collections

Articles on similar topics can be found in the following collections

- Other Endocrinology (624 articles)
- Molecular Medicine (1117 articles)
- Liver, including hepatitis (949 articles)
- Cytopathology (134 articles)

Notes

To order reprints of this article go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to Journal of Clinical Pathology go to:
http://journals.bmj.com/subscriptions/
Comparison of the novel quantitative ARMS assay and an enriched PCR–ASO assay for K-ras mutations with conventional cytology on endobiliary brush cytology from 312 consecutive extrahepatic biliary stenoses

N T van Heek, S J Clayton, P D J Sturm, J Walker, D J Gouma, L A Noorduyn, G J A Offerhaus, J C Fox

Background: Extrahepatic biliary stenosis (EBS) has malignant and benign causes. Patients with EBS are at risk of having or developing malignancy. Accurate diagnostic tests for early detection and surveillance are needed. The sensitivity of biliary cytology for malignancy is low. K-ras mutation analysis on brush cytology is a valuable adjunct, but specificity is low. A quantitative test for K-ras mutations has been developed: the amplification refractory mutation system (ARMS).

Aim: To assess the test characteristics and additional value of ARMS in diagnosing the cause of EBS.

Methods: Brush samples from endoscopic retrograde cholangiopancreatography were collected from 312 patients with EBS. K-ras mutation analysis was performed using ARMS—allele specific amplification was coupled with real time fluorescent detection of PCR products. Results were compared with conventional cytology and K-ras mutation analysis using allele specific oligonucleotide (ASO) hybridisation, and evaluated in view of the final diagnosis.

Results: The test characteristics of ARMS and ASO largely agreed. Sensitivity for detecting malignancy was 49% and 42%, specificity 93% and 88%, and positive predictive value (PPV) 96% and 91%, respectively. The sensitivity of ARMS and cytology combined was 71%, and PPV was 93%. The specificity of ARMS could be increased to 100% by setting limits for the false positives, but reduced sensitivity from 49% to 43%.

Conclusions: ARMS can be considered supplementary to conventional cytology, and comparable to ASO in diagnosing malignant EBS. A specificity of 100% can be achieved with ARMS, which should be considered in the surveillance of patients at risk for pancreatic cancer.

 Differentiating between malignant and benign causes of extrahepatic biliary stenosis (EBS) is often difficult, but very important.1–3 Patients with EBS are at risk of having or developing pancreatic cancer. Others at risk are family members of patients with pancreatic cancer, a fact that has been recognised for decades. Both groups have an increased risk of developing pancreatic cancer, and the application of a screening test for early detection would be very helpful. Because the incidence of pancreatic cancer in the general population is relatively low, screening tests should be limited to the abovementioned groups with increased risk.

"The localisation of most K-ras mutations to the single codon 12 makes them relatively easy to detect" 4

Although the specificity of brush cytology for a malignant cause of EBS obtained during endoscopic retrograde cholangiopancreatography (ERCP) is high,5–8 its sensitivity is relatively low.9 Previously, we showed that the addition of K-ras mutation analysis using allele specific oligonucleotide (ASO) hybridisation to brush cytology improved the sensitivity of diagnosing a malignancy.7 Brush sampling during ERCP has a high probability of yielding sufficient cells for DNA analysis, and these cells may contain mutations that originated in malignant cells preferentially shed from pancreatic ducts. Using these sample, it may also be possible to detect cells originating from other malignant causes of EBS, such as distal bile duct carcinoma, in which K-ras mutations have also been reported.10 The 89% specificity of K-ras mutation analysis based on data from 312 consecutive patients with EBS in our study is one of the highest reported. Nevertheless, a diagnostic test with a specificity of 89% remains suboptimal, especially considering the major therapeutic consequences of a malignant diagnosis.71

Despite its limitations, we think that K-ras is a promising marker in the diagnosis of EBS. The cause of malignant EBS is mostly pancreatic head carcinoma, which has the highest incidence of RAS mutations in human tumours identified to date.12–14 Furthermore, the localisation of most K-ras mutations to the single codon 12 makes them relatively easy to detect.15–16 Depending on the technique used, the reported frequencies of codon 12 mutations range from 20% to 100%, and occur as early events in tumour progression.12 13 17 18

Recently, a novel quantitative real time assay for K-ras mutations has been developed: the amplification refractory mutation system (ARMS) assay.19 20 The real time quantitative approach of this assay, with its sensitive detection and mutant sequence quantification, allows the determination of the true detection limit in any clinical application.20 It routinely provides quantitative data relating to the number

Abbreviations: ARMS, amplification refractory mutation system; ASO, allele specific oligonucleotide; EBS, extrahepatic biliary stenosis; ERCP, endoscopic retrograde cholangiopancreatography; PCR, polymerase chain reaction

See end of article for authors’ affiliations

Correspondence to:
Dr G J A Offerhaus,
Department of Pathology,
Academic Medical Center,
Amsterdam, PO Box 22660, 1100 DD,
Amsterdam, The Netherlands; g.j.
offerhaus@amc.uva.nl

Accepted for publication 17 March 2005

of K-ras mutations in positive samples, allowing a threshold to be set, above which the specificity will be 100%. A quantitative real time assay might be a valuable adjunct to early detection in patients with EB, and also to surveillance strategies in family members of patients with pancreatic cancer. The genetic transmission of pancreatic cancer in hereditary familial syndromes and those patients at high risk are increasingly being better defined. There is a need for a molecular diagnostic test for members of families with certain pancreatic cancer syndromes, which could translate recent molecular genetic discoveries into improved surveillance measures. The ARMS assay for K-ras mutation analysis might be such a test.

The aim of our study was to assess the value of the quantitative ARMS assay for K-ras mutations compared with conventional cytopathology and the established enriched polymerase chain reaction (PCR)–ASO assay for the diagnosis of malignancy in patients with EBS in a large series of consecutive patients with complete follow up.

MATERIALS AND METHODS

Patients

Two earlier studies have been published on the same patient cohort. The study population consisted of 312 consecutive patients who underwent ERCP with endobiliary brush cytology for the evaluation of EBS at the Academic Medical Center in Amsterdam, the Netherlands from January 1993 to February 1996. The mean age of the 312 patients was 63 (range, 19–99) years and the male to female ratio was 173 : 139. The follow up was updated and four additional final diagnoses were encountered that were previously unspecified. For this study, a final diagnosis could be made in 298 patients, 223 (75%) of whom had malignant and 75 (25%) benign stenosis. Table 1 shows the spectrum of the different aetiologies of EBS.

Samples and DNA isolation

The methods for collecting the required samples and isolating the DNA have been described previously. In summary, after brushing of the EBS four cytology smears from each patient underwent conventional Giemsa and Papanicolaou staining and were independently evaluated by an experienced cytopathologist. The following diagnostic categories were used: positive for carcinoma, negative for carcinoma, suspicious for carcinoma, and material insufficient or not suitable for diagnosis. The remaining brush specimen was suspended in 10 ml of DNA buffer, fixed with 10 ml 100% ethanol, and stored at 4°C for subsequent K-ras mutation analysis. The archival tissue blocks, available from 71 patients with malignant EBS and 10 patients with benign EBS, were analysed for K-ras mutations.

A 1 ml aliquot of each brush cytology suspension was used for DNA isolation. Careful microdissection of the tissue blocks was performed to ascertain a sample where at least 50% of the cells comprised the tissue of interest. DNA was extracted as described previously.

K-ras mutation analyses

Two different methods were used for K-ras mutation analysis: the PCR–ASO hybridisation based assay and the novel ARMS allele specific amplification assay.

The protocol for the first method has been described and validated previously. With this assay, DNA is subjected to PCR amplification using primers around codon 12. Digestion of the PCR products with a restriction enzyme is followed by a second round of amplification, which yields a PCR product enriched for K-ras codon 12 mutations. The resulting DNA fragments are denatured and dot blotted onto nylon membranes and subjected to allele specific oligonucleotide hybridisation with radioactively labelled probes specific for each possible K-ras codon 12 mutation, followed by autoradiography. Controls for positive and negative outcomes, contamination, and specific and non-specific hybridisation were used. Both enriched and non-enriched PCR products were dot blotted next to each other to check the digestion and mutant enrichment.

The second method was based on ARMS allele specific amplification for mutant K-ras sequence discrimination. This was undertaken using the ABI 7700 machine (PE Applied Biosystems, Foster City, California, USA) to detect amplification products by fluorescence in real time. This assay has been described in detail. In summary, 5 ml of each of the 11 brush cytology DNA samples was added to each of seven ARMS reactions and a control reaction for DNA in a 96 tube format. An additional control reaction without DNA for each ARMS mix was included to detect possible contamination. The control reaction amplified all K-ras exon 1 sequences, irrespective of mutational status, to measure the total amount of DNA in each sample. Reactions were thermocycled in the ABI 7700 cycler and the relative fluorescence was measured after each cycle. The point at which it exceeded a threshold baseline signal was called the threshold cycle. Threshold cycle values from the control and ARMS reactions were plotted against statistically validated data obtained using wild-type K-ras exon 1. These data were used to establish the background signal resulting from wild-type
K-ras exon 1 over a wide dynamic range of starting DNA concentrations (100 fold). The dynamic range of starting DNA concentrations (6–600 ng DNA) was chosen in relation to the yield of DNA typically obtained from 5 ml of DNA solution extracted from all clinical samples (tissue and cell suspensions in bodily fluids) entering our laboratory. Any clinical sample giving a signal above background in an ARMS reaction had a <1% probability of containing wild-type sequence alone, and was therefore interpreted as positive for that mutation. A similar collection of data was obtained using each of the seven mutant K-ras sequences over the same concentration range. In this way, the amount of K-ras mutant sequence in each positive sample could be calculated as a proportion of the total amount of K-ras exon 1 in that particular sample.

**Definitions of test characteristics**

Sensitivity was defined as the percentage of patients with malignancy and positive test results. Specificity was defined as the percentage of patients without malignancy and negative test results. Positive predictive value was defined as the percentage of patients with positive test results who had a malignancy. Negative predictive value was defined as the percentage of patients with negative test results who did not have a malignancy.

**RESULTS**

Eighty one of 223 (36%) patients with malignant EBS were found to be positive by cytology alone. By combining suspicious cytology results with positive cytology results, 111 of 223 (49%) malignancies could be detected. Ninety four of 223 (42%) patients with malignant EBS were detected by K-ras analysis using the enriched PCR–ASO assay alone, and 109 (49%) were detected by ARMS alone. Table 2 summarises the test results. When the results from the ARMS assay and cytology were combined, 129 of 223 (58%) and 159 of 223 (71%) confirmed malignancies were detected, depending on whether the cytology results were restricted to the positive samples or included the suspicious samples.

The results of the two different methods (the enriched PCR–ASO assay and the ARMS assay) were largely in agreement. Figure 1 shows the frequency of occurrence of each mutation detected by the two assays. The most frequently observed mutations in both assays resulted in codon 12 Gly → Asp and Gly → Val amino acid substitutions. No codon 12 Gly → Ser mutations were detected by enriched PCR–ASO. An additional test for the codon 13 Gly → Asp mutation in the ARMS assay detected eight mutations. There was no equivalent test in the enriched PCR–ASO assay and a zero was recorded for this mutation. Figure 2 shows the ARMS assay results for K-ras point mutations in 100 ERCP samples. Representation by this means has been described previously in lung cancer.20 Most samples in each test were negative for K-ras mutations. The data points appear above the 98% confidence interval established using wild-type K-ras exon 1, or fail to give ARMS products during cycling and are recorded as 50 cycles. Samples appearing below the lower limit of the 98% confidence interval have a <1% chance of containing the wild-type sequence alone and are therefore recorded as positive. Duplicate analysis using the same DNA samples confirmed the positive samples (data not shown).

All three diagnostic methods gave positive results in cases of confirmed non-malignancy. False positivity was 7% (five of 75) for the ARMS assay, 12% (nine of 75) for the PCR–ASO assay, and 3% (two of 75) to 7% (five of 75) for conventional cytology (table 2). Table 3 summarises the results of the 15 samples that had a false positive diagnostic test. Because the results of the ARMS assay were quantitative, cutoff limits were set for each of the four mutations found. These were 12.5%, 0.95%, 1.25%, and 0.24% for the codon 12 Gly → Ala, Gly → Asp, Gly → Cys, and Gly → Val tests, respectively. This means that a specificity of 100% was established for the diagnosis of malignancy using the adjusted ARMS assay. This reduced the sensitivity of the assay from 49% to 43%, because mutations were detected at equivalent or lower levels in samples from patients with clinically confirmed malignancy. Table 4 summarises the sensitivity, specificity, positive predictive value, and negative predictive value for all of the methods used. The main problem with all of the approaches used in isolation was that their negative predictive values were low and they did not detect enough malignancies. However, when ARMS and cytology were combined they were able to detect 159 of 223 (71%) malignancies with a positive predictive value of 93%. Individually, they detected 109 of 223 (49%) and 111 of 223 (50%) malignancies, with positive predictive values of 96% and 94%, respectively.

**DISCUSSION**

Our study shows that the novel quantitative ARMS assay for K-ras mutation analysis is a valuable adjunct to conventional cytology and the non-quantitative PCR–ASO assay in diagnosing EBS. By setting cutoff limits, the ARMS assay was 100% specific and 43% sensitive in diagnosing a malignant cause in 312 consecutive endobiliary brush samples obtained from patients with EBS. Furthermore, a diagnostic combination of conventional cytology and the ARMS assay was able to predict a malignant cause in 93% of patients.

Cytology is highly specific in diagnosing the cause of EBS, but its sensitivity is routinely low.24 Although it has been suggested that repeated brushings increase the sensitivity,25 cytology alone is not sufficient to differentiate between malignant and benign EBS. Our study population comprised patients at risk of having or developing malignancy, mostly...
pancreatic or distal bile duct carcinoma. At present, the early detection of pancreatic cancer is the only realistic option for cure. Although screening for pancreatic cancer in the general population is not justified at the moment, there is certainly a need for accurate diagnostic tests in families with genetic disorders known to predispose to pancreatic cancer. Sensitive molecular assays offer the chance to improve test characteristics when used as an addition to cytology. Obviously, this mainly depends on the prevalence of the candidate marker in the disease and the technique used for the detection of molecular changes.

K-ras mutations are highly prevalent in pancreatic carcinoma and common in bile duct carcinoma, the two main malignant causes of EBS. K-ras encodes a protein located on the inner side of the plasma membrane, which has intrinsic GTPase activity. A mutation in the K-ras gene at codon 12 or 13 results in inappropriate growth signalling. Several studies have investigated the clinical usefulness of K-ras mutation analysis in the diagnosis and treatment of EBS. Reported rates of K-ras mutations and outcomes of the numerous studies vary widely. For example, Ponsioen et al found no additional value of K-ras mutation analysis in
discriminating between benign and malignant strictures in patients with primary sclerosing cholangitis. However, we showed previously that K-ras codon 12 mutation analysis was a useful supplement to light microscopic evaluation of ERCP brush cytology specimens for the diagnosis of malignant EBS.

“Although screening for pancreatic cancer in the general population is not justified at the moment, there is certainly a need for accurate diagnostic tests in families with genetic disorders known to predispose to pancreatic cancer”

Variations in reported diagnostic K-ras mutations can be attributed to the nature of the clinical material being investigated and the sensitivity and specificity of the assays used. In a previous study, we used fine needle aspiration of pancreatic or duodenal juice to show that a molecular panel could still develop malignancy, a diagnostic test with a specificity of 89% remains suboptimal.

The novel real time ARMS assay used in our current study provided quantitative data, allowing the determination of the true detection limit, above which the specificity was 100%. It is a convenient and homogeneous method, which facilitates high throughput sample analysis for a range of clinical materials. The enriched PCR–ASO assay requires separate amplification steps and will always carry the risk of PCR contamination. In contrast, the ARMS assay is a closed tube assay and the amplification products can be disposed of with a vastly reduced risk of contamination. The additional data provided by this sensitive technique used in conjunction with cytology increased the detection of clinically confirmed malignancy from 111 of 223 (50%) to 159 of 223 (71%).

Knowledge about well defined high risk groups that might benefit from sensible surveillance strategies is rapidly increasing. Particularly noteworthy in this regard are the hereditary syndromes that include pancreatic cancer. A

**Take home messages**

- The amplification refractory mutation system (ARMS) for K-ras mutation had a slightly higher sensitivity, specificity, and positive predictive value compared with the polymerase chain reaction–allele specific oligonucleotide assay in diagnosing patients with malignant extrahepatic biliary stenosis (EBS).
- ARMS would be a useful supplement to conventional cytology in diagnosing patients with malignant EBS.
- A specificity of 100% can be achieved with ARMS, and this test should be considered in the surveillance of patients at risk for pancreatic cancer.

---

**Table 3** Summary of results for the 15 non-malignant samples that gave positive results by cytology, PCR–ASO, or ARMS

<table>
<thead>
<tr>
<th>Patient's sex/age (years)</th>
<th>Cytology positive</th>
<th>Cytology suspicious</th>
<th>ASO positive</th>
<th>ARMS positive*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/31</td>
<td>–</td>
<td>12 Asp</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Male/60</td>
<td>–</td>
<td>12 Asp</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Male/43</td>
<td>–</td>
<td>Ala</td>
<td>88 Asl (9%)</td>
<td>–</td>
</tr>
<tr>
<td>Male/46</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Female/85</td>
<td>–</td>
<td>Cys</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Female/44</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Female/54</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Cys (1.25%)</td>
</tr>
<tr>
<td>Male/72</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Male/41</td>
<td>–</td>
<td>12 Asp</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Male/38</td>
<td>–</td>
<td>Ala</td>
<td>Ala (12.5%)</td>
<td>–</td>
</tr>
<tr>
<td>Male/44</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Female/60</td>
<td>–</td>
<td>12 Asp</td>
<td>12 Asp (0.95%)</td>
<td>–</td>
</tr>
<tr>
<td>Male/55</td>
<td>–</td>
<td>–</td>
<td>Val</td>
<td>–</td>
</tr>
<tr>
<td>Male/51</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Female/40</td>
<td>–</td>
<td>12 Asp</td>
<td>Val (0.24%)</td>
<td>–</td>
</tr>
</tbody>
</table>

*Based on a final diagnosis of malignancy, which was established by histological and/or clinical findings (symptomatology, imaging studies, and course of the disease).

ARMS, amplification refractory mutation system; ASO, allele specific oligonucleotide; PCR, polymerase chain reaction.

**Table 4** Summary of the diagnostic specificity and sensitivity of cytology, PCR–ASO, and ARMS in relation to malignancy

<table>
<thead>
<tr>
<th>Diagnostic test result</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology (positive and suspicious)</td>
<td>49%</td>
<td>91%</td>
<td>94%</td>
<td>35%</td>
</tr>
<tr>
<td>Cytology (positive)</td>
<td>36%</td>
<td>97%</td>
<td>98%</td>
<td>39%</td>
</tr>
<tr>
<td>PCR–ASO</td>
<td>42%</td>
<td>88%</td>
<td>91%</td>
<td>34%</td>
</tr>
<tr>
<td>ARMS</td>
<td>49%</td>
<td>93%</td>
<td>96%</td>
<td>38%</td>
</tr>
<tr>
<td>ARMS (adjusted*)</td>
<td>43%</td>
<td>100%</td>
<td>100%</td>
<td>37%</td>
</tr>
<tr>
<td>ARMS</td>
<td>71%</td>
<td>84%</td>
<td>93%</td>
<td>53%</td>
</tr>
</tbody>
</table>

*Adjusted ARMS results based on quantities of K-ras mutant sequence in confirmed non-malignant samples. ARMS, amplification refractory mutation system; ASO, allele specific oligonucleotide; PPV, positive predictive value; PCR, polymerase chain reaction; NPV, negative predictive value.
genetic marker of sufficiently high sensitivity and specificity is required to determine those unaffected relatives in the direct cancer prone lineage who are at increased risk of cancer.\(^1\) The ARMS assay could be a valuable adjunct in this respect, because it can be set to a specificity of 100%. Members of families prone to pancreatic cancer are at high risk for the development of pancreatic cancer and therefore ideal candidates for surveillance.\(^2\) We believe that a symptomatic kindred of patients with a mutation known to be associated with familial susceptibility warrants an aggressive approach that incorporates our growing knowledge of the genetics of pancreatic cancer. The goal for the surveillance of patients with familial pancreatic cancer is to diagnose the malignancy in the dysplasia or carcinoma in situ stage, before the development of invasive cancer. Some authors even advise that a complete pancreatodudenectomy should be performed in these patients.\(^3\) It has already been shown that thorough screening of patients with a family history of pancreatic cancer is feasible.\(^4\) In the near future, other molecular markers of pancreatic cancer, recently discovered by global gene expression technology,\(^5\) could form a suitable diagnostically panel along with K-ras mutation analysis using the ARMS assay on brush cytology.

In conclusion, our study shows that the novel, real time ARMS assay for K-ras mutations could be a useful supplement to conventional cytology and the non-quantitative PCR–ASO assay in diagnosing patients with malignant EBS. The quantitative nature of the ARMS assay makes it possible to diagnose malignant stenosis with a specificity of 100%.

**Authors’ affiliations**

N T van Heek, P D J Sturm, L A Noorduyn, G J A Offerhaus, Department of Pathology, Academic Medical Centre Amsterdam, 1100 DD Amsterdam, The Netherlands

S J Clayton, J Walker, J C Fox, AstraZeneca, Alderley Park, Macclesfield, Cheshire, SK10 4TG, UK

---

**REFERENCES**


www.jclinpath.com

Heek, Clayton, Sturm, et al.