Detection of human papillomavirus in large cell neuroendocrine carcinoma of the uterine cervix: a study of 12 cases

W Grayson, L F Taylor, U Allard, A J Tiltman

Aim: To investigate the role of human papillomavirus (HPV) in large cell neuroendocrine carcinoma (LCNEC) of the uterine cervix.

Methods: Twelve archival, immunohistochemically and/or electron microscopically confirmed cases of cervical LCNEC were studied. Non-isotopic in situ hybridisation (NISH) was performed on the formalin fixed, paraffin wax embedded biopsies using digoxigenin labelled probes to HPV types 6, 11, 16, 18, 31, and 33. The tumours were then subjected to polymerase chain reaction (PCR) analysis using GP5+/GP6+ consensus primers to the HPV L1 gene, in addition to type specific primers to the E6 and E7 genes.

Results: HPV-16 was detected by NISH and/or PCR in seven of the 12 carcinomas. Two additional tumours were HPV-18 positive by NISH and/or PCR. HPV DNA was not detected in the three remaining cases.

Conclusion: Integration of high risk HPV, in particular type 16 and to a lesser extent type 18, is associated with this uncommon variant of cervical carcinoma.

Large cell neuroendocrine carcinoma (LCNEC) of the uterine cervix is an uncommon, clinically aggressive malignant neoplasm, with fewer than 50 cases documented in the literature. It is generally accepted that integration of high risk HPV into the host genome is the single most important event in the evolution of cervical cancer. There is a well documented association between high risk HPV infection and cervical intraepithelial neoplasia (CIN), squamous cell carcinoma, adenocarcinoma, adenosquamous carcinoma, and rare variants of cervical cancer, including adenoid cystic carcinoma, adenoid basal carcinoma, and recently malignant mixed Mullerian tumours of the uterine cervix. Although there is a known association between HPV-18 integration and small cell carcinoma of the cervix, only a few cervical LCNECs have been the subject of molecular investigation for HPV infection. Our present series, the largest to date, explores the role of high risk HPV infection in the evolution of this rare, possibly underdiagnosed, variant of cervical cancer.

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METHODS

Biopsy material

Twelve formalin fixed, paraffin wax embedded biopsy specimens of cervical LCNEC were retrieved from the archival surgical and consultation files of our department. Parallel sections from each case were stained with haematoxylin and eosin, and the histological diagnoses reviewed using recognised morphological criteria.

Immunohistochemistry

Further parallel sections were cut for the purposes of immunohistochemical confirmation of neuroendocrine differentiation. The sections were incubated with antibodies to broad spectrum cytokeratin (MNF 116), synaptophysin, and chromogranin A. Table 1 lists the sources and dilutions of the antibodies used. The presence of paranuclear dot-like immunostaining with MNF 116, in combination with positive staining for synaptophysin and/or chromogranin A, was considered to be diagnostic of neuroendocrine differentiation in these large cell neoplasms.

Electron microscopy

Additional biopsy material was obtained from cases 3 and 11, and submitted in a solution of 3% paraformaldehyde and 0.1% glutaraldehyde. The specimens were postfixed in 2% osmium tetroxide in Millonig’s buffer, followed by dehydration with ethanol and subsequent embedding in Spurr’s resin.

Table 1: Immunohistochemical stains used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNF 116</td>
<td>Dako, Glostrup, Denmark</td>
<td>1/100</td>
<td>StreptABC/HRP (Duet)</td>
</tr>
<tr>
<td>Antisynaptophysin</td>
<td>Dako, Glostrup, Denmark</td>
<td>1/20</td>
<td>StreptABC/HRP (Duet)</td>
</tr>
<tr>
<td>Antichromogranin A</td>
<td>Dako, Glostrup, Denmark</td>
<td>1/100</td>
<td>ABC</td>
</tr>
</tbody>
</table>

ABC, avidin biotin complex; HRP, horseradish peroxidase.

Abbreviations: CIN, cervical intraepithelial neoplasia; HPF, high power field; HPV, human papillomavirus; LCNEC, large cell neuroendocrine carcinoma; NISH, non-isotopic in situ hybridisation; PCR, polymerase chain reaction; TBT, Tris buffered saline.
tissue was sectioned on an ultratome and stained with a saturated solution of uranyl acetate and Reynold's lead citrate. The sections were examined on a Hitachi H-600 transmission electron microscope.

Non-isotopic in situ hybridisation (NISH)

NISH was performed using a previously described technique. Briefly, 4 µm thick sections obtained from each paraffin wax embedded tumour sample were placed on to slides pretreated with aminopropyltriethoxysilane (Sigma, St Louis, Missouri, USA), allowed to dry overnight at 42°C, and then dewaxed and rehydrated according to a standard protocol. The slides were treated with 3% hydrogen peroxide in methanol to reduce non-specific peroxidase activity. Unmasking of nucleic acids was facilitated by limited proteolysis in proteinase K (500 µg/ml) at 37°C. The reaction was stopped in distilled water after 15 minutes. The slides were then dried, before the addition of 6 µl aliquots of hybridisation mix containing 2 ng/µl of digoxigenin labelled HPV types 6, 11, 16, 18, 31, or 33 (obtained from Professor CS Herrington, Liverpool University, UK). Drying was prevented by the application of a coverslip on to each section. The slides were then placed in a moist Petri dish. Target DNA and probe were allowed to hybridise at 42°C for two hours. Sections were denatured in a hot air oven at 95°C for 15 minutes, and then dewaxed and rehydrated according to a standard protocol. The sections were subjected to two posthybridisation washes of five minutes each in 4× standard saline citrate buffer, followed by incubation in TBT (Tris buffered saline containing 3% (wt/vol) bovine serum albumin and 0.05% (vol/vol) Triton X-100) for 20 minutes. Conventional immunohistochemical techniques were used for the detection of hybridised probe. The sections were incubated for 30 minutes with monoclonal antidigoxigenin (1/2000 dilution; Sigma), followed by biotinylated rabbit antimouse F(ab')2 fragment (1/200 dilution; Dako, Glostrup, Denmark) for 30 minutes. Final incubation was in avidin–peroxidase conjugate (1/75 dilution; Dako) containing 10 M dNTP, 1 U Tag polymerase (Boehringer Mannheim, Randburg, South Africa), and 0.8 µM each of the GP5+ and GP6+ primers. Each 50 µl reaction for the E6 and E6/E7 amplification contained 10mM Tris/HCl (pH 8.3), 50mM KCl, 5.5mM MgCl2, 200µM dNTP, and 1.25 U Taq polymerase (Boehringer Mannheim, South Africa), and 0.25µM each of the E6 or E6/E7 primers. Forty cycles of a three step amplification for HPV L1 were performed on a Perkin Elmer thermocycler according to the following protocol: denaturation for four minutes at 94°C, followed by 40 cycles of incubation at 94°C for one minute, 40°C for two minutes, and 72°C for 1.5 minutes. This was followed by a five minute extension period at 72°C. The PCR products was digested under the following conditions: denaturation for four minutes at 94°C, followed by 30 cycles of incubation at 94°C for 0.5 minutes, 55°C for two minutes, and 72°C for two minutes. This was followed by a seven minute extension period at 72°C.

Aliquots of 15 µl of each amplification product were analysed for the expected product size of 150 bp (HPV L1), 228–268 bp (HPV E6/E7 for virus types 16, 18, 31, 33, 35, 52b, and 58), 140 bp (HPV E6 for virus types 16, 18, and 33), 141 bp (HPV E6 for virus type 33), and 268 bp (β-globin) on 3% agarose gels stained with ethidium bromide. For the E6/E7 based typing, the PCR products were digested with restriction enzymes (Acc I, Alu I, Ava II, and Bgl II), followed by agarose gel electrophoresis. Separate gels were used for the L1, E6, E6/E7, and β-globin analyses. The molecular weight of the products was determined with DNA molecular weight marker V (pBR 322 DNA cleaved with HAE III; Boehringer Mannheim, South Africa). Extracted DNA from paraffin wax sections known to contain HPV DNA was used as a positive control. Two negative control tubes were set up using the same PCR method as above. Extracted sample DNA was omitted from one tube, whereas DNA extracted from tissue not harbouring HPV DNA was added to the second of the tubes. Gels were viewed with an ultraviolet transilluminator, and

<table>
<thead>
<tr>
<th>Primer</th>
<th>5′→3′ nucleotide sequence</th>
<th>Target</th>
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<tbody>
<tr>
<td>GP5+</td>
<td>TTTGTTACCTGGTGTAGATTACATC</td>
<td>HPV L1</td>
</tr>
<tr>
<td>GP6+</td>
<td>GAAAAATAAATCGTGAACATACATTC</td>
<td>HPV L1</td>
</tr>
<tr>
<td>HPVpE</td>
<td>AAGGCCTCATAACGGAAATCTGCT</td>
<td>HPV E6</td>
</tr>
<tr>
<td>HPV16R</td>
<td>GTTTCAGCTCTGTGCATGA</td>
<td>HPV E6</td>
</tr>
<tr>
<td>HPV18R</td>
<td>GTGTGCTACCTGTCGCCAG</td>
<td>HPV E6</td>
</tr>
<tr>
<td>HPV33K</td>
<td>GTCTCAAACGTGCGCCAGCACCA</td>
<td>HPV E6</td>
</tr>
<tr>
<td>HPVpLI3M</td>
<td>GTCCAAACCGGTGTTGCTCC</td>
<td>HPV E6/E7</td>
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<tr>
<td>HPVpLI3B</td>
<td>GTGATACCTGGTGTACCTG</td>
<td>HPV E6/E7</td>
</tr>
<tr>
<td>HPVpLI2R</td>
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<td>HPV E6/E7</td>
</tr>
<tr>
<td>GH20</td>
<td>AAAGGACCAAGCAGGATGCC</td>
<td>β-Globin</td>
</tr>
<tr>
<td>PC04</td>
<td>CAATCCATACACGGTCCACC</td>
<td>β-Globin</td>
</tr>
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</table>

The NISH signal pattern was evaluated using the criteria of known to contain the specific HPV types under investigation.

Polymerase chain reaction (PCR)

Two 10 µm tissue sections from each biopsy were applied to a clean glass slide. Strict PCR protocol was adhered to at all times to prevent contamination and cross contamination of samples. Sections were dewaxed, rehydrated, rinsed in sterile distilled water, and allowed to air dry. DNA was extracted from the samples using the Qiagen QIAmp tissue kit (Qiagen Ltd, Dorking, UK). In summary, the samples were digested overnight in a lysis buffer containing proteinase K (Boehringer Mannheim, Mannheim, Germany) at 55°C, followed by precipitation of DNA with ethanol in a spin column. Finally, the DNA was eluted with distilled water. Three separate primer sets were used for the detection of HPV 17-21. These included GP5+/GP6+ primers to the HPV L1 gene, an HPV E6 based detection set for HPV types 16, 18, and 33 (Takara Shuzo, Saga, Japan), and an E6/E7 based typing set for the detection of both benign (HPV-6 and HPV-11) and malignancy associated viral types (HPV types 16, 18, 31, 33, 35, 52b, and 58) (Takara Shuzo). Table 2 lists the primer sequences used. A volume of 10 µl of each DNA sample was amplified using each of these primer sets. Parallel amplification of the β-globin gene (GH20 and PC04) was performed to determine the integrity of the DNA in each sample (table 2). Each 50 µl reaction for the L1 amplification contained 10mM Tris/HCl (pH 8.3), 150mM KCl, 3.5mM MgCl2, 200µM dNTP, 1.25 U Taq polymerase (Boehringer Mannheim, Randburg, South Africa), and 0.5µM each of the GP5+ and GP6+ primers. Each 50 µl reaction for the E6 and E6/E7 amplification contained 10mM Tris/HCl (pH 8.3), 50mM KCl, 1.5mM MgCl2, 200µM dNTP, and 1.25 U Taq polymerase (Boehringer Mannheim, South Africa), and 0.25µM each of the E6 or E6/E7 primers. Forty cycles of a three step amplification for HPV L1 were performed on a Perkin Elmer thermocycler according to the following protocol: denaturation for four minutes at 94°C, followed by 40 cycles of incubation at 94°C for one minute, 40°C for two minutes, and 72°C for 1.5 minutes. This was followed by a five minute extension period at 72°C. The PCR products was digested under the following conditions: denaturation for four minutes at 94°C, followed by 30 cycles of incubation at 94°C for 0.5 minutes, 55°C for two minutes, and 72°C for two minutes. This was followed by a seven minute extension period at 72°C.

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Table 2 PCR primer sequences used for the human papillomavirus (HPV) L1, E6, E6/E7, and β-globin genes

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photographed on instant black and white film (Fujifilm, Tokyo, Japan).

RESULTS
Clinical material
Age was known in 11 of the 12 cases, and ranged from 42 years to 74 years (mean, 59.6). Eleven patients were black and one was white.

Light microscopy
All 12 biopsy specimens showed a high grade malignant neoplasm composed of relatively large cells with moderate amounts of cosinophilic cytoplasm, ill defined intercellular borders, and large pleomorphic vesicular nuclei with irregular nuclear contours and prominent nucleoli (fig 1). Most of the cases exhibited a predominantly solid, trabecular and organoid growth pattern, with vague peripheral palisading by tumour cells within the neoplastic islands. Ill defined pseudorosettes were encountered in five cases. All of the lesions were characterised by extensive surface ulceration and geographical areas of tumour necrosis. Two tumours also contained much smaller neoplastic epithelial islands reminiscent of those encountered in adenoid basal carcinoma. Microscopic foci of malignant squamous differentiation were seen in three cases. There was brisk mitotic activity, with all 12 neoplasms showing in excess of 10 mitoses/10 high power fields (HPFs). In some lesions, there were in excess of 13 mitoses in a single HPF. Small foci of residual, non-ulcerated, benign surface squamous epithelium were present in five cases, whereas in case 8 there was evidence of CIN III. Case 8 was composed predominantly of large, solid basaloid islands with peripheral palisading. Several of the tumour cells in cases 1 and 9 contained cosinophilic intracytoplasmic granules, a phenomenon that has been described previously in LCNEC of the cervix. None of the tumours showed an accompanying squamous cell carcinomatous or adenocarcinomatous component. In case 6, however, there was a minor adjacent component of small cell neuroendocrine carcinoma. An unusual feature in case 3 was the focal presence of small extracellular cylinders of non-Congophilic basement membrane-like material. These microscopic areas were morphologically reminiscent of similar foci described in the

Figure 1  High power photomicrograph of large cell neuroendocrine carcinoma of the cervix showing characteristic trabecular morphology. Note large, mitotically active tumour cells with evidence of focal palisading.

Figure 2  Positive immunostaining with MNF 116. Note characteristic paranuclear dot-like accentuation of the staining pattern in this typical large cell neuroendocrine carcinoma.

Figure 3  Diffuse immunostaining for synaptophysin in large cell neuroendocrine carcinoma, confirming the presence of neuroendocrine differentiation in this cervical neoplasm.

Figure 4  Electron photomicrograph of pseudorosette in large cell neuroendocrine carcinoma, case 10 (uranyl acetate and lead citrate; original magnification, x5250).
solid variant of adenoid cystic carcinoma of the cervix. Elsewhere, however, the appearances were more typical of LCNEC.

Immunohistochemistry
Neuroendocrine differentiation was confirmed immunocytochemically in all but one case, namely, case 10. The biopsy in case 10 was completely non-immunoreactive, a phenomenon attributed to prolonged fixation. Additional material from this case was thus submitted for ultrastructural confirmation of neuroendocrine differentiation. However, the 11 immunoreactive tumours showed diffuse positive staining with MNF 116 (fig 2), and moderate to intense immunostaining for synaptophysin (fig 3). There was characteristic paranuclear dot-like accentuation of the cytokeratin staining pattern with MNF 116. None of the lesions was positive for chromogranin A.

Electron microscopy
Ultrastructural examination of biopsy material from cases 3 and 10 confirmed the presence of large pleomorphic epithelial cells with well developed intercellular desmosomes. The irregular nuclei were pleomorphic, with granular chromatin and one or more prominent nucleoli. Pseudorosettes were encountered in case 10 (fig 4). The cytoplasm of the tumour cells in both lesions contained glycogen and numerous scattered dense core neurosecretory granules (fig 5), confirming the presence of a carcinoma with neuroendocrine differentiation. In case 3, there were extremely focal areas with an adenoid cystic carcinoma-like ultrastructural appearance, characterised by neoplastic epithelial cells enveloping small cylinders of redundant basal lamina (fig 5). Amyloid fibrils were not identified.

NISH
The NISH signal pattern was assessed according to the criteria of Cooper et al. Table 3 summarises the NISH results. Five of the 12 tumours were found to harbour integrated HPV-16

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Synopsis of human papillomavirus (HPV) status of 12 cervical large cell neuroendocrine carcinomas by NISH and PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case No.</td>
<td>HPV type</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
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</tr>
<tr>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
</tr>
</tbody>
</table>

HPV types 6, 11, 31, and 33 were not detected with NISH.
*HPV types 6, 11, 31, 33, 52b, and 58 were not detected with this restriction endonuclease digestion system; †HPV type 33 not detected.

NISH, non-isotopic in situ hybridisation; PCR, polymerase chain reaction.
DNA, as shown by the presence of punctate (type 2) signals in three lesions, and admixed type 2 and type 3 (combined punctate and diffuse) nuclear signals in two cases (fig 6). One case showed type 2 NISH signals for HPV-18. Type 1 (diffuse) signals indicative of episomal HPV DNA were not encountered in any of the cases. Furthermore, there were no signals for HPV types 6, 11, 31, or 33.

**DISCUSSION**

Over the years, much terminological confusion has surrounded endocrine neoplasms of the uterine cervix. This has resulted in a plethora of diagnostic terms, making it difficult to determine the incidence, histological criteria, clinical behaviour, and optimal treatment for lesions that form part of the spectrum of cervical neuroendocrine tumours. This is compounded by the fact that although the World Health Organisation’s classification of uterine cervical neoplasms recognises carcinoid tumour and small cell carcinoma of the cervix as specific entities, there is a conspicuous omission of lesions such as atypical carcinoid tumour and LCNEC from that classification. In 1996, a workshop was convened under the auspices of the College of American Pathologists and the National Cancer Institute to clarify these issues. A new classification was proposed that encompasses four entities; namely, typical (classic) carcinoid tumour, atypical carcinoid tumour, large cell neuroendocrine carcinoma, and small (oat) cell carcinoma. This classification scheme is identical to that used for pulmonary neuroendocrine neoplasms, and uses the same diagnostic criteria for each of the entities. Criteria for the diagnosis of cervical LCNEC include the presence of large cells with vesicular nuclei and prominent nucleoli, a mitotic rate of at least 10/10 HPFs, geographical areas of tumour necrosis, and positive staining with appropriate neuroendocrine markers. All of the cases in our series fulfilled these diagnostic criteria, although one case (case 10) was non-immunoreactive and required ultrastructural confirmation of the light microscopic diagnosis of LCNEC.

“It is quite possible that LCNECs are frequently misdiagnosed as poorly differentiated squamous cell carcinomas or poorly differentiated adenocarcinomas”

LCNEC of the cervix is now recognised as a distinct clinicopathological entity. Although fewer than 50 cases have been reported, it has been suggested that the lesion may not be as uncommon as was originally thought. It is quite possible that LCNECs are frequently misdiagnosed as poorly differentiated squamous cell carcinomas or poorly differentiated adenocarcinomas, based upon the identification of focal areas of squamous or glandular differentiation, respectively. In such cases, the subtle neuroendocrine features of the large cell neoplasm are easily overlooked. In our present series, one tumour showed microscopic and ultrastructural evidence of basement membrane-like material, resulting in morphological overlap with the rarely reported solid variant of cervical adenoid cystic carcinoma. Two additional lesions contained microscopic basaloid islands similar to those observed in cervical adenoid basal carcinoma. Therefore, it is apparent that the accurate diagnosis of cervical LCNEC is dependent not only on an adequately sized biopsy specimen, but also a high index of suspicion. Accurate diagnosis of this uncommon form of cervical cancer is of prognostic importance. Recent studies have reaffirmed the biologically aggressive nature of LCNEC. Based on their data and an extensive review of the literature, Gilks et al identified a 65% mortality within three years of diagnosis, with frequent extra-abdominal metastases. Patients with stage I LCNEC also have a poor outcome. In a recent review of 21 cases of stage I LCNEC, 12 patients had died of disease after a median survival period of 16 months. The mortality rate thus appears to be similar to that of small cell cervical carcinoma. Furthermore, occasional examples of cervical small cell carcinoma with a variable component of LCNEC have been reported in the literature, and one LCNEC (case 6) in our series harbourd a microscopic area of small cell carcinoma. Therefore, a close histogenetic relation seems to exist between large cell and small cell neuroendocrine carcinomas. For many years, pulmonary pathologists endeavoured to separate small cell carcinomas of the lung from non-small cell pulmonary neoplasms, largely for therapeutic reasons. In recent years, however, it has become apparent that both small cell and large cell forms of neuroendocrine bronchogenic carcinoma exist, and that both represent aggressive histological subtypes. This issue was dealt with in a recently published proposal for the updated terminology of neuroendocrine neoplasms. According to this classification, both tumour types are simply designated grade 3 neuroendocrine carcinoma, whereas classic carcinoid and atypical carcinoid are
regarded as grade 1 and grade 2 neuroendocrine carcinomas, respectively. Whether or not this terminology will gain the acceptance of gynaecological pathologists, however, still remains to be seen.

Only three previous studies have explored the role of HPV in LCNEC (table 4). The first such study was by Mannion et al in 1998. This study comprised 38 cases, of which five were classified as pure LCNEC, and a further three were classified as small cell carcinoma with focal LCNEC. The role of HPV was investigated by NISH in 10 of the 38 tumours. Although it was stated that eight of 10 cases showed punctate staining with the HPV-16/18 probe, a shortcoming of this study is the fact that the exact number of HPV positive LCNECs was not mentioned specifically. Of the eight HPV-16/18 positive tumours also showed rare punctate signals with the HPV-31/33 probe. Wistuba et al used PCR to investigate 15 neuroendocrine tumours of the cervix for the presence of HPV DNA, including two LCNECs. One of the two LCNECs was found to harbour HPV-18, whereas the other case was PCR negative. Yun et al reported a case of cervical LCNEC in which integrated HPV-16 was demonstrated by NISH, not only within the LCNEC component, but also within the overlying CIN III.

It has been suggested that in addition to viral exposure, host factors or other non-viral factors may play a role in the evolution of cervical cancer. Several recent publications have focused on the role of the p53 tumour suppressor gene and the short arm of chromosome 3 in both neuroendocrine and non-neuroendocrine cervical neoplasms. We were able to demonstrate integrated HPV-16 and, to a lesser extent, HPV-18 (both high risk HPV types) in a large number of the LCNECs in our present series. However, the demonstration of high risk HPV integration is an almost ubiquitous finding in most cervical carcinoma subtypes, yet LCNEC is an uncommon variant despite its confirmed association with HPV. It is plausible that both HPV integration and loss of heterozygosity at 3p loci may be required for the evolution of cervical neuroendocrine carcinomas of both small cell and large cell type. It is also curious that although LCNEC is an aggressive neoplasm associated with high risk HPV types (16 and 18), adenoid basal carcinoma of the cervix, another HPV-16 associated neoplasm, generally pursues an indolent clinical course. However, rare examples of adenoid basal carcinoma of the cervix may exhibit divergent differentiation and show more aggressive growth. The additional role played by host factors at a genetic level in all histological subtypes of cervical cancer remains to be fully elucidated, and clearly warrants further investigation.

ACKNOWLEDGEMENT

This study was supported by grants from the SA Institute for Medical Research, and the HE Griffin Trust administered by the University of the Witwatersrand. The clones used in the preparation of the probes for NISH were obtained from Dr E-M de Villiers, Papillomavirus Referencezentrum, Heidelberg, Germany (HPV types 6, 11, 16, 18), Dr AF Lorincz, Digene Diagnostics, Silver Spring, Maryland, USA (HPV 31), and Professor C Orth, Institut Pasteur, Paris, France (HPV-33). The authors thank Mrs G King for the electron photomicrography, and Messrs M Lanesman and G Hall for their assistance with the photomicrography.

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H Holzel, P van Diest
Efficient isolation of campylobacters from stools: what are we missing?

The past three decades have witnessed the rise of campylobacter enteritis in humans from virtual obscurity to notoriety, with present isolation rates superseding those of other enteric pathogens such as Salmonella spp. and Shigella spp. in most developed countries. Unlike the salmonellae and other enteric pathogens, most (>99%) clinical reports concerning campylobacter are sporadic, and campylobacter enteritis outbreaks are rare. Although campylobacters are not completely new to applied bacteriology, they have largely evaded traditional techniques used for the isolation of pure cultures, apart from single isolations that were free from competing organisms. Until the development of a selective medium by Skirrow,1,2 these organisms were known mainly by veterinarians as animal pathogens, which were responsible for a wide variety of disorders in cattle, sheep, and pigs.3 Since the development of more sophisticated isolation techniques, the true disease potential of these organisms has become apparent and today campylobacteriosis is regarded as a zoonosis, which is capable of being transmitted to humans by a wide range of domestic animals.4

There have been several reports describing the inability of selective media to recover certain species of campylobacter, especially the catalase weak or negative organisms, from faecal specimens.5 In addition, there are large epidemiological differences between rates of infection with campylobacters between Northern Ireland and the rest of the UK; however, no data exist with regard to the rates of isolation of the atypical campylobacters from stool specimens locally. Therefore, it was the aim of our study to evaluate the efficacy of recovery of clinically relevant campylobacters from faecal specimens in Northern Ireland, using direct plating and differential filtration techniques.

One hundred and eighty six faecal specimens from an equal number of patients with acute gastroenteritis were examined for the presence of Campylobacter spp. over the peak seasonal period, May to June. Faecal specimens were obtained from family practitioners in the community and were examined within 24 hours of receipt. Two isolation methods were compared for their efficacy in recovering viable organisms, namely: (1) direct plating of faeces on to two selective media at two different incubation temperatures (37°C and 42°C); and (2) differential filtration on non-selective medium incubated at 37°C. For both treatments, faeces were emulsified in 0.1% (wt/vol) peptone water. For direct plating, 10 μl of faecal suspension was inoculated on to both modified CCDA agar (Oxoid Ltd, Poole, Dorset, UK), containing cefoperazone (0.2 mg/litre) and amphotericin B (10 mg/litre), which was subsequently incubated at 37°C, and also on to Preston’s selective medium (Oxoid Ltd), containing rifampicin (10 mg/litre), trimethoprim (10 mg/litre), cycloheximide (100 mg/litre), and polymyxin B (5000 IU/litre), which was incubated at 42°C. For recovery by differential filtration, 300 μl of faecal suspension was passively filtered through a 0.65 μm cellulose triacetate membrane (Millipore, Edinburgh, UK), and incubated at 37°C, as described previously.6 In both treatments, plates were incubated in microaerophilic conditions (5% (vol/vol) O2) for two to five days. Presumptive positive colonies were further characterised as described previously.7 Direct plating, 18 of 186 (9.7%) faecal specimens were positive, whereas 22 of 186 (11.8%) specimens were positive by the differential filtration method using non-selective media (table 1). All campylobacters that grew on one or other selective medium were also isolated by differential filtration, except for Helicobacter fennelliae, which was only isolated by non-selective filtration. The use of both media together missed thermophilic campylobacter in three specimens, which were positive by filtration. Statistical analysis was performed using a paired Student’s t test and this gave a probability of p = 0.0226, demonstrating a significant difference between differential filtration and selective plating techniques.

Overall, our study shows that non-thermophilic campylobacters were not commonly isolated from faeces and that the use of a combination of selective media was superior to the use of one selective medium only, and that use of differential filtration with a non-selective medium was superior to direct plating on selective agars. Surprisingly, only one atypical organism, H. fennelliae, was isolated from 186 patients, and direct plating failed to detect up to six strains of Campylobacter jejuni. Similar to our study, in England and Wales, the infectious intestinal diseases study8 noted remarkably few cases of other organisms, including Campylobacter upsaliensis, Campylobacter fetus, Campylobacter hoerinenteralis, and Campylobacter lariense; whenever a filtration method of selective media was used. However, this study did not comment on the number of C jejuni strains missed by selective plating, which was significant in the Northern Ireland study. Given that approximately 1000 laboratory reports for campylobacters from faeces in Northern Ireland are currently received by the Communicable Disease Surveillance Centre (Northern Ireland) annually, extrapolation of recovery rates based on our study would suggest approximately 27 cases being undetected in the laboratory.

Most clinical laboratories in the UK isolate campylobacters from stools at incubation temperatures of greater than 40°C, usually 42–43°C. In our study, we noted a slightly higher isolation rate for Preston’s selective agar incubated at 42°C, compared with the CCDA medium, which was incubated at 37°C, where an additional three specimens were positive using the former technique. Previously Bolton et al noted that a higher recovery rate was made from CCDA medium at 37°C as opposed to 42°C.9 Although the Public Health Laboratory Service standard operating procedure for the investigation of faeces for the presence of campylobacters10 recommends an incubation temperature of 35–37°C for primary isolation, perhaps the use of a temperature of greater than 40°C would be better.

Moore and Murphy16 previously demonstrated that the use of selective agents in laboratory media may result in the failure to recover sensitive strains. In our present study, the inability of the selective media used to recover all strains might result from the sensitivity of these wild-type C jejuni and H fennelliae to the antibiotics incorporated within the selective formulations.

Presently, in Northern Ireland, most clinically relevant campylobacters from faeces are being isolated on either Preston’s or Skirrow’s

| Table 1 | Comparison of recovery of campylobacters from human stools using selective plating versus non-selective differential filtration techniques |
|-----------------------------------------------|
| Isolation technique (incubation temperature) | Total no. faeces examined | Number of specimens positive (%) positive | Species identified |
|-----------------------------------------------|
| Differential filtration (37°C)                | 186 | 22 (11.8%) | 21 Campylobacter jejuni |
| CCDA selective agar (37°C)                    | 186 | 15 (8.1%)  | 1 Helicobacter fennelliae |
| Preston’s selective agar (42°C)               | 186 | 18 (9.7%)  | All C jejuni |
|                                              |     |             | All C jejuni |

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### Evidence for antibiotic induced *Clostridium perfringens* diarrhoea

We read with interest the paper entitled “Evidence for antibiotic induced *Clostridium perfringens* diarrhoea”. The authors review the current knowledge of this syndrome and discuss the need for routine screening. To contribute to this debate we present the results of a recent survey performed in our laboratory. *Clostridium difficile* is a major cause of antibiotic associated diarrhoea (AAD) but an increasing number of reports implicate *C perfringens* as a cause of this condition.

Because our hospital has a substantial number of cases of AAD, we decided to perform a survey of the incidence of *C perfringens* enterotoxin in stools from hospital inpatients.

Over a six month period, all inpatients who presented with loose or watery stools submitted to the microbiology department were included in our study. Anyone who had tested positive for *C difficile* or *C perfringens* within the previous six months for anyone who had already been enrolled as a case was excluded, so that only incident cases were investigated. For all cases, a detailed questionnaire was completed to try to ascertain the risk factors predisposing to AAD caused by either of these two pathogens.

Each stool sample was tested for *C difficile* toxins A and B in addition to *C perfringens* enterotoxin using commercially available enzyme linked immunosorbent assay kits (Becton, Lab, Blacksburg, USA). All samples were also processed for other bacterial pathogens using standard methods. Of the 249 samples tested, 24 (9.6%) were positive for *C difficile* enterotoxin; however, of these, only four (1.6%) were positive for *C perfringens* toxin. None of the samples was positive for both *C difficile* and *C perfringens* toxins and no other bacterial pathogens were isolated.

Of the 24 *C difficile* positive patients, 18 were over 60 years of age. Half of the positive patients had clinical diarrhoea (more than three loose stools each day). Eighteen of the 24 had received some antibiotic treatment, with fluclaxacillin and cefuroxime being the most frequently used, either singly or in combination with other antibiotics. Only one patient had received clindamycin. The presence of severe or disabling underlying disease was reported in 17 of the 24 patients. Five positive patients received antibiotic treatment with metronidazole.

All of the four patients with *C perfringens* toxin were women, their respective ages were 55, 72, 92, and 94. Two were in medical wards and the other two were from renal wards. Only one was recorded to have clinical diarrhoea. Of the positive patients, three had disabling disease and only one had antibiotic treatment before developing diarrhoea. None of the positive patients required antibiotic treatment.

*Clostridium perfringens* enterotoxin has been implicated as a cause of antibiotic associated diarrhoea and diarrhoea by person to person transmission in hospitalised patients, and also in elderly patients, not related to food borne outbreaks. Another possible route of transmission is orally ingested spores from the environment or staff members in hospitals. In our study, only four (1.6%) patients were *C perfringens* toxin positive and only one of these had clinical diarrhoea. As Modi and Wilcox recognise, there are considerable resource implications associated with routine screening for *C perfringens* enterotoxin. The apparent low incidence of *C perfringens* enterotoxin in patients with loose stools and the relatively mild symptoms displayed by positive patients suggests that routine screening may not be justified in our hospital.

### References


The journal apologises for the omission of H A Rhemtula from the list of authors on the first page of this paper. The list of authors should have read as follows: H A Rhemtula, K J Perry, G Ford, D J funky, G Majumdar, P K Gould.