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Title	Accuracy of diagnostic testing in primary ciliary dyskinesia
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Publication date	2016-02-29
Original Citation	Jackson, C. L., Behan, L., Collins, S. A., Goggin, P. M., Adam, E. C., Coles, J. L., Evans, H. J., Harris, A., Lackie, P., Packham, S., Page, A., Thompson, J., Walker, W. T., Kuehni, C. and Lucas, J. S. (2016) 'Accuracy of diagnostic testing in primary ciliary dyskinesia', European Respiratory Journal, 47(3), pp. 837-848. doi: 10.1183/13993003.00749-2015
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1183/13993003.00749-2015
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Download date	2026-11-07 03:35:17
Item downloaded from	https://hdl.handle.net/10468/9259



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Supplementary Methods

Nasal nitric oxide measurement

Nasal nitric oxide (nNO) was measured using NIOX® Flex (Aerocrine, Sweden). The chemiluminescence analyser was maintained, calibrated and tested according to manufacturer's guidelines. Ambient NO was recorded and patient measurements made by trained healthcare professionals. Measurements were made in patients aged >5 years and capable of breath-hold for 20 seconds. A nasal probe sampled gas aspirated from the nostril at 0.3 L/min and measurements were recorded during simple breath-hold from a steady nNO concentration plateau of at least 3 seconds. Maximum nNO was acquired from triplicate measurements which received plateau state. The highest recording was used. Nasal NO concentration in parts per billion (ppb) were converted to production rate in nL/min by multiplying ppb by the flow rate (L/min). We used nNO as an adjunct test rather than screening test for PCD with a cut-off of <30 nl/min making a positive diagnosis very likely and >77 nl/min to make diagnosis less likely. nNO measurements were not available at satellite centres, but for difficult cases patients were invited to attend UHS for nNO and re-testing if needed.

Nasal brush biopsy

Nasal epithelial cells acquired by nasal brushing biopsy using 2 mm diameter cytology brushes (Olympus EndoTherapy BC-202D, KeyMed, UK), were submerged in 1.5 ml Medium 199 (Gibco, Invitrogen, UK) with additional antibiotics (50 units/ml penicillin and 50 µg/ml streptomycin). Couriered samples were submerged in a 2.5 ml medium for high-speed video microscopy microscopy (HSVM) and/or 3% glutaraldehyde for transmission electron microscopy (TEM).

High-speed video microscopy

Nasal epithelium in suspension (100 µl) was enclosed by coverwell chamber 20 mm diameter x 0.6 mm depth (GBL635051, Grace Bio-labs, Sigma, UK) onto a glass microscope slide and warmed to 37^{o}C in an environmental chamber (Solent Scientific, UK) to equilibrate for approximately 30 minutes. HSVM analysis was performed within 6 hours of sampling and cells were imaged on an Olympus IX71 inverted microscope using an Olympus x100 UPlanFL objective lens with 1.30 numerical aperture with oil used non-conventionally with a replacement Olympus U-PCD condenser from an upright microscope which provided phase contrast conditions. A Photron FASTCAM- MC2 high-speed video camera (Photron, UK) captured ciliary beating at 500 frames per second (fps) for digital analysis using Photron PFV v3.1.7.1 Camera Control Software at 30 fps. CBF and CBP were analysed by trained PCD Scientists: Mean CBF [(500/ frames for 10 ciliary beats) x 10] from a minimum of 6 independent ciliated strips (normal range, 11 to 20 Hz (20)); CBP was qualitatively assessed and defined as normal or dyskinetic (static cilia, uncoordinated cilia, rotational cilia, reduced beat amplitude, slow or hyperfrequent ciliary function). High speed video microscopy analysis was considered normal only if both CBP and CBF were normal. Equivocal results or abnormal results led to recommendation for repeat sampling or reanalysis following ALI-culture.

Transmission electron microscopy

Nasal epithelium fixed in 3% glutaraldehyde was prepared for TEM using standard protocols and micrographed at 60,000x magnification (minimum) by Hitachi H7000. Typically 300 cilia in transverse and some longitudinal section (100 cilia minimum if 'normal') were systematically analyzed avoiding repeat analysis, unhealthy cilia, or images of the cilium

apex and base. Quantitatively, “9+2” microtubular arrangement and the presence of dynein arms and radial spokes was assessed. Defects included absence or shortening of ODA and/or IDA dynein arms, disarranged, absent or transposed microtubules. Extra microtubules and compound cilia were considered to be secondary defects. Cilia in longitudinal sections were investigated for transposed outer microtubule doublets. Cilia orientation was confirmed as unidirectional or not. Tissue was evaluated qualitatively for yield and health of the epithelium. Although it is part of our diagnostic protocol to have “hallmark PCD defects” on two occasions, it does not improve diagnostic accuracy and our protocol needs changing to reflect that one TEM analysis is sufficient if the ultrastructural changes are clear-cut.

Air-liquid interface culture

Nasal epithelium was cultured in specialised medium at an air-liquid interface (ALI) until differentiated as previously described (14). Briefly, nasal epithelial cells were re-suspended in bronchial epithelial growth medium (BEGM) supplemented with SingleQuots (Clonetics, Lonza, UK) and additional 0.5 units/ml penicillin and 500 ng/ml streptomycin (Gibco, Invitrogen, UK) and 0.2 units/ml nystatin (Sigma-Aldrich, UK) and seeded on 300 µg/ml collagen (PureCol, Nutacon, NL). Cells were incubated at 37^{o}C in 5% CO_2 at 100% relative humidity. Epithelial basal cells were grown to approximately 90% confluent in one well of a 12-well culture plate and passaged to a collagen coated T-25 cm^2 culture flask. Subsequently, cells were passaged at 90% confluence and seeded 1x10^5 basal epithelial cells onto collagen coated 12 mm diameter, 0.4 µm pore transwell inserts (Costar, Fisher Scientific, UK) and once confluent exposed to an air-liquid interface with ALI differentiation medium containing 1:1 DMEM 4.5 g/L D-glucose (Gibco, Invitrogen, UK):BEGM with

additional 100 nM all-trans retinoic acid (Sigma-Aldrich, UK) fed basolaterally. Medium was replaced three times per week and ciliation occurred from approximately 3 weeks post ALI culture. The degree of ciliation was estimated to range between 5-50% by low power light microscopy (20x objective lens). For HSVM or TEM analysis ciliated cells were routinely prepared by excision of a segment of transwell membrane (containing cells) or by gently scraping cells from the surface of the transwell membrane to provide a cell suspension. HSVM and TEM analysis was conducted as described above.

Statistical Analysis

The distribution of demographic, clinical history, neonatal history and clinical symptoms were examined by univariate analysis. Prevalence of categorical variables was presented as percentages, and Chi-squared and Fishers exact tests assessed proportional differences. For continuous variables mean ± standard deviation (SD) with two tail parametric (t) or non-parametric (x2, Mann-Whitney) tests were presented. P value <0.05 was considered statistically significant.