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Unlocking Spatial Molecular & Cellular Relationships of SARS-CoV-2 in Archived Human Tissue

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DISCLAIMER

This protocol was originally developed March-September 2020, and will be updated as further clinical and pathological evidence of SARS-CoV-2 infection are published.

ABSTRACT

The true prevalence of SARS-CoV-2 infection in the UK population remains unknown. Phylogenetic diversity estimates derived from genomic sequence tracking algorithms suggest possible arrival of viral strains in the UK several weeks earlier than the first confirmed cases. Diagnostic pathological specimens have continued to be recovered from patients throughout the Covid-19 pandemic, as part of standard investigation and workup across the normal range of clinical indications. Given the symptomatic heterogeneity and probable high rate of asymptomatic infections, we hypothesise that tissue biopsies have already been taken from living patients with clinically unrecognised SARS-CoV-2-infections. Here, we describe the protocol for a retrospective cohort study with the aims of i) detecting SARS-CoV-2 using qRT-PCR in archived biopsy tissue recovered from patients over the course of the pandemic to date, ii) demonstrating qualitative and quantitative evidence of SARS-CoV-2 tissue tropism and cellular-molecular patterns of infection using histological, in situ hybridisation and immunohistochemical techniques, and iii) linking those spatial molecular-cellular characteristics of SARS-CoV-2 infection to clinico-pathologic parameters. The results will provide new insights into the tissue tropism and rates of asymptomatic and 'atypical' SARS-CoV-2 infections - information that could immediately impact healthcare practices or public health policy.

ATTACHMENTS

covid19tissueprotocolv210920.pdf

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Unlocking Spatial Molecular & Cellular Relationships of SARS-CoV-2 in Archived Human Tissue

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Keywords

SARS-CoV-2, COVID-19, RT-PCR, RNA in situ hybridisation, molecular mechanism, formalin-fixed paraffin-embedded tissues

[4-6 keywords]

Abstract

The true prevalence of SARS-CoV-2 infection in the UK population remains unknown. Phylogenetic diversity estimates derived from genomic sequence tracking algorithms suggest possible arrival of viral strains in the UK several weeks earlier than the first confirmed cases. Diagnostic pathological specimens have continued to be recovered from patients throughout the Covid-19 pandemic, as part of standard investigation and workup across the normal range of clinical indications. Given the symptomatic heterogeneity and probable high rate of asymptomatic infections, we hypothesise that tissue biopsies have already been taken from living patients with clinically unrecognised SARS-CoV-2-infections. Here, we describe the protocol for a retrospective cohort study with the aims of i) detecting SARS-CoV-2 using qRT-PCR in archived biopsy tissue recovered from patients over

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[193 words (double spaced)]

Introduction

The clinical spectrum of SARS-CoV-2 infection remains under intense investigation (Figure, verity_figure.pdf), with the proportion and nature of asymptomatic infections still being explored[1,2].To date no studies have directly attempt to identify asymptomatic cases of SARS-CoV-2 infection in human tissue samples derived from living patients. Estimates of the probable rates of asymptomatic infections from latest large retrospective population-based studies suggest these infections may account for up to a third of cases[2].Rates described from smaller prospective screening cohorts have demonstrated variable results to date, and such studies are limited to selected populations being screened (healthcare workers, returning travellers, hospital cohorts)[3,4,5,6,7], with no possibility to examine 'pre-lockdown' rates of infection patients.

Figure adapted from Verity et al 2020, showing probable clinical distribution of SARS-CoV-2 cases; The dotted outlines representing the target population in this study and potential to identify asymptomatic and atypical clinical cases.

Uncertainty also remains around the earliest phases of the outbreak, with the precise routes and pace of spread into and amongst the UK population still speculative and based on genomic sequence data. Phylogenetic divergence events within different SARS-CoV-2 strains[8], extrapolated from analysis of viral genomic sequences from the earliest cases identified, suggest the virus could have been within the UK population several weeks prior to the first diagnostically confirmed cases. With no population screening in these early phases of the outbreak, we must look to other sources of patient material to identify SARS-CoV-2 infections within this possible window of emergence. Any such 'historical' infections could yield viral genomic sequence data that would improve existing molecular evolutionary data, tighten confidence intervals on temporal inferences of phylogenetic models, and potentially improve tracking of the virus within the population.

Characterisation of SARS-CoV-2 tissue tropism across the spectrum of possible clinical presentations is also yet to be comprehensively reported. Tissue tropism of a viral pathogen can be postulated by receptor distribution, and ACE2 expression (host receptor for both the SARS-CoV and SARS-CoV-2 viruses[9]) has been well characterised[10]. In the 2003 SARS-CoV outbreak, active infection was demonstrated within pneumocytes of the lower respiratory tract but also, crucially, in glandular-type epithelial cells of non-respiratory tissues, including small intestinal and gastric mucosa. Gastro-intestinal symptoms may feature in ~10% of hospitalised SARS-CoV-

2 patients[11]. Most studies of severe coronavirus infection in human tissue have been limited to post-mortem studies with small cohort sizes, therefore only capturing the histological features of severe cases. To date, the majority of 'antemortem' tissue studies have been limited descriptive histological case reports, with only a handful providing incomplete virologic correlation or characterisation (eg RT-PCR, IHC and ISH)[12-58] *(NB this literature review includes only articles with PMIDs, and excludes dermatologic manifestations, was last updated on 29/07/2020, and will be updated as new PMID publications relevant to the protocol are identified)*. Tissue from asymptomatic individuals and individuals with 'atypical' clinical presentations may be of great significance to public health policy, may help inform diagnostic criteria for recognition of SARS-CoV-2 infection, and may provide insight into the natural history of the pathogen.
[313]

Approach
The natural history of SARS-CoV-2 infections, particularly in non-severe clinical contexts (eg asymptomatic, atypical or mild presentations, that comprise the majority of infections within the population), remains largely unknown. Surgical and diagnostic tissue samples have continued to be recovered from patients throughout the time-period of emergence of SARS-CoV-2, as part of standard investigation, workup and treatment across the standard range of clinical indications, from routine surveillance of chronic conditions through urgent elective investigation and management of cancers to emergency admissions with acute medical and surgical presentations. We hypothesise that tissue samples may have been taken from patients infected with SARS-CoV-2.These tissue samples could act as an untapped, pre-existing bank of patient material collected throughout the early phase of the Covid-19 outbreak, and representing a patient population that may include asymptomatic and atypical cases, who would not otherwise have been tested or clinically recognised (Figure). In this project, we aim to screen this tissue for presence of SARS-CoV-2, and characterise the histopathological and molecular features of SARS-CoV-2 infection.
[170]
Aims and objectives
We propose to undertake a retrospective cohort study with the aim of screening for, and characterising SARS-CoV-2 infection in archived FFPE tissue samples recovered from patients as part of routine standard of care over a probable window of emergence of the virus within the UK population.

PHASE I: SCREENING
Identification & screening of FFPE tissue cohorts for SARS-CoV-2
Diagnostic testing of patients within NHS Lothian is currently directed at symptomatic patients and known contacts of symptomatic patients. The test itself is based on a quantitative reverse transcriptase PCR assay worked up by Public Health England that amplifies a short specific region of the SARS-CoV-2 RdRp gene (see Methods for details).RNA is preserved within formalin fixed tissue, and routinely recovered from FFPE tissue samples for molecular testing in NHS Lothian, with methods recovering sufficient material for next generation sequencing applications. While the diagnostic test for SARS-CoV-2itself has not yet been validated for use in FFPE tissue sample-derived RNA, there is strong precedence for both PCR-based amplification of RNA targets from FFPE tissue samples, and for detection of viral nucleic acids from FFPE tissue samples.

(i)**Aim: Create a cohort of tissue samples taken throughout the probable emergence window of SARS-CoV-2 and establish the likely prevalence of SARS-CoV-2 within the cohort by PCR screening**
(ii)**Objective:** identify all archived FFPE tissue samples with sufficient remaining material, recovered from patients

as part of routine standard of care in NHS Lothian, and submitted and processed by the pathology department in NHS Lothian from 01/01/2020 to the present; extract RNA from sections taken from each tissue sample within the cohort, and screen the extracted RNA for the presence of SARS-CoV-2 viral RNA using the RdRp quantitative reverse transcriptase PCR assay; estimate the prevalence of SARS-CoV-2 within the cohort, as a proportion of the total screened cases and total screened patients with appropriate statistical confidence limits
(iii)**Hypothesis:** SARS-CoV-2 viral RNA can be detected in a proportion of archived FFPE tissue samples equal to the estimated population prevalence

PHASE II: CHARACTERISATION

Molecular & spatial characterisation of putative SARS-CoV-2 tropism

Tropism of a viral pathogen can be postulated by receptor distribution in host cells, tissues and organs, or inferred by demonstration of the presence of viral RNA by PCR in tissue-derived RNA. Neither of these, however, provide demonstration of the specific cells and tissues infected or damaged by a virus, or, crucially, of active viral RNA synthesis/replication in infected cells. Formalin fixation preserves tissue and cellular architecture, as well as both nucleic acids and proteins within their native subcellular locations. In situ hybridisation and immunohistochemistry can be used to directly visualise viral and host RNA and proteins within the context of tissue and cellular structure in FFPE tissue samples.

RNAScope™ (a state-of-the-art in situ hybridisation technology) provides the potential not only to detect presence of positive-strand viral genomic/subgenomic RNA (using an *antisense* probe) within cells in FFPE tissue, but also to detect presence of negative-strand viral RNA (using a *sense* probe). Owing to the replication strategy of coronaviruses, the presence of *any* negative-strand viral RNA (ie non-genomic RNA not present in the virion) detected by presence of sense ISH signal provides evidence of i.) transcription & translation of viral genomic RNA (to generate the viral replicase complex responsible for synthesis of negative-strand viral RNAs) and ii.) activity of the viral replicase complex in an infected cell. Ergo, *sense* ISH signal (as a surrogate marker of viral RNA synthesis) should only be detected in the presence of *antisense* ISH signal in cells, enabling classification of such *two signal positivecells* as being capable of supporting at least a degree of viral transcriptional activity and replicase activity (albeit not definitive evidence of productive viral replication). In contrast, the presence of *antisense* ISH signal ***in the absence*** of sense ISH signal may indicate either i.) an early (pre-transcriptional) stage of infection, or ii.) entry of a cell *impermissible* to viral transcription and/or replication. This enables classification of all cells in tissue into three viral infection 'states': absence of antisense signal & sense signal (=*no* viral infection); presence of antisense signal / absence of sense signal (=*extant* viral infection); presence of antisense signal **&** sense signal (=*active* viral infection).

The outputs of RNAScope™ have the potential for (i) absolute quantification (continuous data), (ii) ordinal semi-quantitative scoring, (iii) comparative ratios of extant:active infection or (iv) threshold data. Different tissue types may have differing signal patterns (that cannot currently be predicted with confidence) that would warrant different analysis approaches. As such we will pick the appropriate quantification & analysis strategy for the appropriate expression pattern. Indeed, to limit bias this analysis may be complementary (in conjunction with 'manual' histological examination) to automated counting using digital image analysis.

(i)**Aim: Characterise putative SARS-CoV-2 tropism at molecular, cellular and tissue levels in PCR-positive samples**
(ii)**Objective:** visualise the presence of SARS-CoV-2 positive-strand genomic/subgenomic RNAs & negative-strand

intermediaries, and presence of select host transcripts (ACE2, TMPRSS2, IFNbeta) in PCR-positive FFPE tissue samples using a state-of-the-art in situ hybridisation technique, analysed by a combination of manual histological examination and digital image analysis; visualise the presence of SARS-CoV-2 components and host receptors/entry-associated proteases at the protein level using immunohistochemistry on the same (dual stained) tissue sections; classify cells with ISH-confirmed SARS-CoV-2 infection by the presence of extant versus active viral infection, by specific cell type and tissue type; quantify (using appropriate analysis as outlined above) viral & host transcript levels per cell (augmented where appropriate by digital image analysis); examine variance of viral & host transcript levels, ratios of extant:active infection, extant infection:host transcripts & active infection:host transcripts at cell type-, tissue type- and organ/gross anatomical part-level
(iii)**Hypothesis:** putative SARS-CoV-2 cellular and tissue tropism is associated with expression of established host receptors/transmembrane proteins

PHASE III: CORRELATION

Exploring correlation of viral load with active viral infection in SARS-CoV-2-positive tissues

The clinical and pathogenic importance of viral load remains under intense investigation in SARS-CoV-2 patients. Viral load may have potential as a marker for severity, infectivity or prognosis of infection, and previous coronavirus pandemics have demonstrated correlation between clinical manifestations and site-specific viral load. Viral load can be estimated from the outputs of the diagnostic RdRp qRT-PCR in RNA samples tested for SARS-CoV-2. At present, the relationship between viral load and *active* viral infection of cells in tissues has not been demonstrated for SARS-CoV-2.

(i)**Aim: Correlate PCR-derived viral load to ISH-derived evidence of extant & active viral infection at organ-/gross anatomical part-level**
(ii)**Objective:** examine for evidence of correlation between estimated viral load (derived from established PCR-based quantification of viral RNA per ml of total RNA from FFPE tissue) and level of active versus extant viral infection in the same sample (derived from ISH-based quantification of *antisense* versus *sense* ISH signals in FFPE tissue)
(iii)**Hypothesis:**viral load correlates withthe degree of active viral infection in tissues

Exploring correlation of clinico-pathological parameters with SARS-CoV2 organ tropism

Tropism can be considered a dynamic state between virus and host. While host receptor expression may be sufficient for attachment and entry of a virus, a multitude of other cellular, tissue and systemic factors also influence tropism, and ultimately the ability of the virus to cause disease. Clinical and histological parameters can be used as surrogates for systemic, organ and tissue factors that might influence the distribution and propagation of a virus in the host. Pre-existing database linkage of archived FFPE tissue samples to unique patient identifiers provides a means to link any of the molecular or histological outputs from this study to a panel of basic clinical information. Clinico-pathologic correlation may, therefore, enable characterisation of SARS-CoV-2 tropism beyond the tissue level, and elucidate the clinical context of any cases identified in this study, potentially shedding light on atypical or asymptomatic forms of infection.

(iv)**Aim: Correlate presence of active SARS-CoV-2 infection at organ-/gross anatomical part-level to demographic & clinical parameters**
(v)**Objective:** Examine for evidence of correlation between presence of active viral infection (as defined above) in organs/gross anatomical parts and a panel of pre-defined, known demographic (age; sex) and clinical (procedural

& clinical context of tissue removal; co-morbidity profile; immunosuppression status; relevant drug history; date & result of any SARS-CoV-2 diagnostic test) parameters
(vi)**Hypothesis:** putative SARS-CoV-2 tropism is determined by host/clinical factors beyond receptor expression

PICOS framework
Population:
-any patients who have had any tissue removed as part of standard of care for any purpose (=tissue samples) within NHS Lothian, where that tissue has subsequently been submitted and processed, in part or in whole, as a formalin-fixed paraffin-embedded specimen (=FFPE tissue samples) in the NHS Lothian pathology department, and is surplus to diagnostic requirements (=archived FFPE tissue samples) from 01/01/2020 to date.

Intervention (=Exposure):
-SARS-CoV-2-infected human tissue; demonstrated by i.) presence of viral RNA by PCR amplification from RNA extracted from archived FFPE tissue samples AND ii.) visualisation of viral RNA by in situ hybridisation in cells in archived FFPE tissue samples.

Comparison:
-SARS-CoV-2-negative human tissue & cases; demonstrated by absence of viral RNA (by PCR amplification and in situ hybridisation) in archived FFPE tissue samples.

Outcome measure(s):
-demonstration of putative tropism of SARS-CoV-2 infection across a broad range of clinical and host contexts.
-identification of 'new historical' SARS-CoV-2 cases during the earliest phases of the outbreak.
-demonstration of pathogenic characteristics and host pathologic response to SARS-CoV-2 infection in specific tissue & cell types.

Further predefined subgroup aims & analyses
The following aims are defined here as proposed subgroups that are largely dependent on the outputs of the primary aims, which will determine the subgroup sample sizes and/or technical feasibility of the experiments involved. Further amendments and/or separate protocols will be authored for each subgroup as their individual feasibility of the proposed experiments becomes more certain.

Sequencing of 'new historical' SARS-CoV-2 viral genomes
Current phylogenetic modelling of the evolution of SARS-CoV-2 (derived from genome sequencing data) and mapping of spread and subsequent evolution within geographically distinct populations is predominantly based on virologic swab-derived patient material. Any positive cases identified in tissue in this project could represent 'new historical' strains of SARS-CoV-2 during the critical period of emergence of the virus, with a defined 'date stamp', and amenable to submission to the national COVID-19 Genomics UK (COG-UK) sequencing consortium. Any 'new' cases will provide a unique retrospective source of sequence data with the potential to strength (tighten confidence intervals) or alter evolutionary models and, by extension, understanding the spread of SARS-CoV-2.

(i)Aim: sequence any 'new' identified cases of SARS-CoV-2 derived from archived FFPE tissue samples
(ii)Objective: identify 'new' cases, defined as patients with a PCR-positive FFPE tissue sample, but no recorded

positive SARS-CoV-2 virologic swab result (data already collected within the remit of the project); via local Tissue Governance administration, transfer RNA and required meta-data from 'new' cases (requiring de-identification by the Tissue Governance administration) to the COG-UK consortium for sequencing
(iii)Hypothesis: a proportion of the PCR-positive SARS-CoV-2 cases represent 'new historic' viral strains
Feasibility limits; number of positive samples, capacity within the COG-UK project for sequencing of 'non-diagnostic' samples

Comparison of 'known' nasopharyngeal-derived SARS-CoV-2 viral genome sequence to tissue-derived viral genome sequence
Alongside the variation in clinical presentation of SARS-CoV-2 infection, there is variation in the duration of infections, and patterns of viral shedding across different clinical contexts is unknown. Current phylogenetic evidence suggests the mutation rate of SARS-CoV-2 may be relatively low. Pre-existing database linkage of archived FFPE tissue samples to unique patient identifiers provides a means to link any PCR-identified virus in FFPE tissue back to any diagnostic virologic swab-confirmed SARS-CoV-2 cases in the same patient (eg a patient with both a positive tissue sample and a positive nasopharyngeal swab result). This could provide the opportunity to sequence virus from the same patient in two distinct organ/gross anatomical parts, and/or potentially at different timepoints. As significant sequence differences would not be anticipated, any detectable genomic divergence may indicate infection with a 'second'/non-related strain.

(i)Aim: sequence any 'known' identified cases of SARS-CoV-2 derived from archived FFPE tissue samples *and* from the corresponding patient's diagnostic nasopharyngeal swab
(ii)Objective: identify 'known' cases, defined as patients with a PCR-positive FFPE tissue sample, *and* a recorded positive SARS-CoV-2 virologic swab result (data already collected within the remit of the project); via local Tissue Governance administration, transfer RNA and required meta-data from these 'known' cases (requiring de-identification by the Tissue Governance administration) to the COG-UK consortium for sequencing; compare sequence data from nasopharyngeal 'strain' to tissue 'strain'
(iii)Hypothesis: SARS-CoV-2 genomic sequence does not vary between site of infection
Feasibility limits; number of positive samples, capacity within the COG-UK project for sequencing of 'non-diagnostic' samples

Transcriptional characterisation of putative SARS-CoV-2 tropism through virus-host next generation sequencing
In situ hybridisation enables high resolution mapping of individual viral and host transcripts with cellular- and tissue-level resolution, but the technique is currently unfeasible for substantial multiplex profiling beyond a handful of targets. NanoString nCounter® is a state-of-the-art digital molecular transcriptomic technology capable of quantification of hundred targets within FFPE tissue-derived RNA, that can now include probes for detection and quantification of SARS-CoV-2, with potential for distinguishing extant versus active infection (using *antisense* and *sense* probes, as outlined above).

(i)**Aim: Characterise putative SARS-CoV-2 tropism by profiling viral and host transcriptional activity in ISH-confirmed PCR-positive samples**
(ii)**Objective:** measure viral and host gene expression (focussing on host receptors, entry-associated transmembrane proteins, inflammatory, apoptotic and innate immune-related targets) in RNA extracted from FFPE tissue samples with ISH-confirmed presence of extant or active SARS-CoV-2 infection using a state-of-the-art next generation sequencing platform, and in RNA extracted from tissue- & organ-matched FFPE tissue

samples with ISH-confirmed absence of viral infection; compare transcriptional profiles between tissues with no viral infection, extant viral infection and active viral infection
(iii)**Hypothesis:** putative SARS-CoV-2 tropism is associated with the transcriptional profile of host tissues
Feasibility limits; sufficient remaining material (FFPE tissue) and quality of RNA following completion of primary aims for transcriptional profiling, subgroup sizes by viral infection state

Classification & correlation of histological features of SARS-CoV-2 infections to tissue type & clinical parameters
The histological features of host response that we may observed in any SARS-CoV-2-positive cases are largely unknown in asymptomatic, mild and atypical cases. However, aspects that are likely to be assessable include: composition and severity of any inflammatory responses within tissues with infected cells; nature of any reactive or cytopathic effects in infected (or neighbouring) cells; interface between any 'background' pathological processes (that may be co-incident with the identified infection) sites of infection. Furthermore, these histological features may be observed at subcellular-, cellular-, tissue- and/or organ/gross anatomical part-levels. Owing to the largely unknown nature of any such changes, it is not possible to fully anticipate relevant groups for analysis based on the possible combinations of location, nature and clinical context within which histological changes might occur. The outputs from this study may, however, enable classification of infections on the basis of these histological features, and therefore provide the foundation for future studies in any emerging cohorts. Digital image analysis technologies, in combination with 'manual' histological examination, can be used by pathologists to annotate, analyse and quantify histological features and the outputs of tissue-based molecular assays, potentially providing reproducible and semi-automated classification methods. To this end, the following subgroup analysis is proposed.
(i)Aim: characterise the histological features of SARS-CoV-2 infection in FFPE tissue samples
(ii)Objective: within each organ/gross anatomical part, characterise 'cellular' histological features of infection by presence/nature of any reactive/cytopathic effects in (ISH-confirmed) infected cells, and cells within a local radius (for solid-type tissues) or trajectory distance (for surface-type tissues) from infected cells; characterise 'inflammatory' histological features by presence/composition/severity of any inflammation within a radius/trajectory from ISH-confirmed site of infection and within infected tissue compartments as a whole; classify 'background' pathological entities (where feasible) and characterise proximity of infected cells to background pathological entities; examine relationships between any identifiable groups of histological features (presently unknown) of SARS-CoV-2 infection and site of infection (cell type/tissue type/organ) and clinical parameters
(iii)Hypothesis: histological features of SARS-CoV-2 infection are specific to tissue and organ type, and to the clinical context of infection
Feasibility limits; subgroup sizes by organ, tissue & cell type, technical capabilities of digital image analysis for non-biased assessment of histological features correlated against manual histology outputs

Correlation of viral infection state (extant versus active), cell type-/tissue type-specific infection and clinical parameters
It is not possible to predict the patterns or distribution of viral infection (eg extant infection versus active infection), or the range and hierarchy of specific cell types and tissue types that may harbour SARS-CoV-2 in this study. Subgroups, defined by common observed combinations of pattern of viral infection across specific cell types and/or within specific tissue types may emerge from Phase II analysis and may be amenable to correlation with clinical parameters as broadly outlined in Phase III analysis.

(i)Aim : correlate combinations of viral infection state (extant/active) and/or cell type- or and/or tissue type- and/or organ/gross anatomical part-specific infection to demographic and/or clinical parameters
(ii)Objective: Examine mutual relationships between any emerging subgroups and a panel of pre-defined, known demographic (age; sex) and clinical (procedural & clinical context of tissue removal; co-morbidity profile; immunosuppression status; relevant drug history; date & result of any SARS-CoV-2 diagnostic test) parameters
(iii)Hypothesis: putative SARS-CoV-2 tropism is determined by host/clinical factors beyond receptor expression
Feasibility limits; subgroup sizes by organ, tissue, cell type, & viral infection state

Study design
This is a retrospective cohort study, using archived FFPE tissue samples to screen for the presence of SARS-CoV-2 RNA. All tissue samples identified within the study window from a database search will be subject to;
-histology review to establish presence of sufficient tissue for viral screening,
-RNA extraction from subsequent FFPE sections,
-quantitative reverse transcriptase PCR screening of FFPE-extracted RNA for SARS-CoV-2 RdRp gene, consistent with the approach undertaken for routine nCOV2019 testing in the Department of Laboratory Medicine,
with any PCR-positive cases subject to;
-in-depth histological and molecular phenotyping using dual in situ hybridisation (RNAScope®) and immunohistochemistry, with probes directed against SARS-CoV-2 Spike gene (sense and antisense probes, to enable delineation of active and extant infection), ACE2, TMPRSS2 and IFN-beta, and antibodies directed against ACE2 (receptor), TMPRSS2, CTSB and CTSL (entry-associated proteases) and IFN-beta,
-in silico analysis of spatial virus-to-host relationships, generating a final cohort of deeply phenotyped SARS-CoV-2 tissue infection cases.

Tissue & patient selection rationale
Given the evolving evidence base for clinical features of infection, tissue tropism, and the possibility of presence of secondary receptors (as demonstrated in the 2003 SARS-CoV secondary receptor CD209L), this protocol has been designed to allow future exploration of tissue type that might not currently be recognised clinically or pathologically as 'relevant' to SARS-CoV-2 infection.

The study will use de-identified (=pseudoanonymised) archived (=surplus to diagnostic needs) FFPE tissue samples. We do not intend to select patients on the basis of any clinical information, beyond the act of removal of tissue (and submission and processing of that tissue within Pathology) as part of routine standard of care within NHS Lothian. Our intent with this approach is an attempt to capture the broadest possible representative population of patients (accepting a degree of co-morbidity heterogeneity owing to the need for a tissue-based procedure), the majority of whom will have been in the community at time of tissue removal, and to screen tissues that would not routinely be tested for SARS-CoV-2, and also screen patients who may not otherwise have been screened (unless Covid19 infection was suspected). There is precedence for this approach in previous population-scale tissue-based studies, with the retrospective screening of appendix and tonsillar specimens for prion protein, without any specific cohort selection, that provided invaluable insight into the natural history and likely outcome of the vCJD outbreak.

Study window and expected prevalence rationale
The study window is based on phylogenetic divergence models suggesting possible earlier timepoints for 'index' infection events out with the original Chinese epicentre. The first diagnostically-proven cases of SARS-CoV-2

within the UK were confirmed on 31/01/2020. Phylogenetic diversity tracking of SARS-CoV-25 demonstrate a genomic divergence event occurring within a hypothesised window of 06/01/2020 to 26/01/2020, that generated many of the genomic lineages from which the largest cluster of UK-identified strains have evolved. While the geographic location of this divergence point (which must correspond to at least one infection event) cannot be established, it could suggest arrival of 'index' strains within European populations, possibly within the UK, several weeks earlier than the first diagnosed cases.

Estimates of asymptomatic and atypical symptomatic infections are speculative, however current models suggest UK prevalence ranging from ~1 to 6%. We therefore anticipate 10 to 60 positive cases for every 1000 screened cases.
[540]
[200-400 words]

Methods
Cohort definitions
The study window is defined as 01/01/2020 to the present. Within this study, a 'case' is defined as a tissue sample, or series of tissue samples, removed from any patient as part of the routine standard of care, for any purpose, during a single healthcare encounter within NHS Lothian (corresponding to a single procedure/intervention on a single date), where that tissue has subsequently been submitted and processed, in part or in whole, as a formalin-fixed paraffin-embedded specimen within the NHS Lothian pathology department, and is deemed surplus to diagnostic requirements.

Each case may include more than one individual FFPE tissue sample, and may include more than one tissue type (for example, a patient undergoes an endoscopic examination of the upper gastro-intestinal tract; two distinct anatomical sites are biopsied and submitted in two separate containers as two 'parts' (=tissue samples) in the same case, which, upon submission to pathology are processed as two separate FFPE blocks (=FFPE tissue samples). No cases will include tissue from more than one patient or tissue from more than one date/healthcare encounter. Individual patients may have had more than one 'case' submitted and processed by NHS Lothian during the study window (for example, a patient has a diagnostic biopsy of part of a lesion, followed several weeks later by removal of the lesion as a surgical excision), therefore some patients will have tissue samples screened for SARS-CoV-2 over more than one time point across the study window. By virtue of linkage of the unique case identifier to a unique patient identifier (Community Health Index number, or CHI) any patients with multiple cases over time can be readily identified and annotated as such within the cohort, to ensure this is accounted for in any downstream statistical analysis (eg to avoid 'double counting' in any patient- or clinical-focussed analysis).

The study cohort is therefore ultimately composed of cases, each corresponding to a single dated episode where at least one tissue sample has been removed from a patient, submitted and processed into a corresponding FFPE tissue sample.

Case identification and ethical approval for use in research
NHS Lothian's Department of Laboratory Medicine maintains a Laboratory Information Management System (LIMS) database of all pathology specimens, in a format that can be searched by date (each case being date-stamped), tissue type (using SNOMED codes linked to each case at time of authorisation), sample type (using

LIMS codes linked to each case to distinguish diagnostic, cytology & surgical specimens). The LIMS database can therefore be searched to identify and stratify (as per screening strategy outlined below) all cases submitted and processed during the study window. This list will then be passed to the Lothian NRS BioResource, which holds ethical approval from the East of Scotland Research Ethics Committee (reference number 15/ES/0094) to release tissue samples and linked patient data from surplus diagnostic material held within the Department of Laboratory Medicine.

Case review for tissue sufficiency

While the majority of cases will already have at least one 'original' haematoxylin & eosin(H&E)-stained slide (generated as part of routine standard of care), additional sections may have been taken from the FFPE block subsequent to this 'original' slide during routine work-up of the case (eg to provide additional H&E-stained levels, or for IHC or molecular analyses). We therefore plan to use a 'new' slide taken from the archived FFPE block for each tissue sample, which will provide a more representative view of the tissue remaining in each case.From the case lists generated by LIMS database searches, the BioResource can provide a 'new' H&E-stained 5 micron section from each FFPE tissue sample in each case, for histological review of tissue sufficiency. Prior to delivery to the pathologists, each case can be de-identified by the BioResource during preparation of the 'new' H&E-stained sections. The BioResource will maintain a key to enable subsequent linkage of specific clinical information (see below) for those cases deemed sufficient for screening. Donor anonymity will be upheld at all times and no sample shall be supplied by the BioResource to the research team which could result in the original donor being identified.

Sufficiency is defined as a total tissue area on one 5 micron H&E of not less than 5 mm^2, or for FFPE samples comprised of cells derived from fluid or aspirate of a solid organ, not less than 100 nucleated cells across a minimum of 15 high power fields examined across one 5 micron H&E. Each 'new' H&E-stained section will be screened by a pathologist for sufficiency, with those deemed sufficient re-screened by a second pathologist for agreement, and re-screened by a third pathologist in the event of disagreement over total tissue area.

Exclusion criteria for cases will therefore be the absence of sufficient material, defined as either i.) tissue less than 5 mm^2 in total area or, ii.) cells (in FFPE) derived from body fluids numbering less than 1500 cells on one 5 micron section (eg 3000 cells in one 10 micron section of tissue), as agreed by at least two pathologists based on review of the 'new' H&E for each FFPE tissue sample in each case. The list of cases with sufficient FFPE tissue can then be returned to the BioResource as the 'screening cohort'.

Clinical data linkage

The BioResource holds ethical approval to provide samples with linked anonymised clinical data where required (as described, by virtue of the existing routine linkage of unique case identifiers with the unique patient identifier, CHI). Using CHI to search NHS Lothian's electronic health record system (Trak), a panel of basic clinical parameters will be derived for each FFPE tissue sample in each case in the screening cohort, to include;
-organ/gross anatomical part from which tissue removed (eg head & neck excluding skin & CNS, respiratory tract & mediastinum, thoracic cavity not already stated, lymph nodes & spleen, upper gastro-intestinal tract, lower gastrointestinal tract, hepatopancreatobiliary tract, renal & urinary tract, genital tract, abdominal cavity not already stated, placental tissue, bone & peripheral soft tissues, central nervous system including eye, skin)[SOURCE: Labs IM&T\coded information from case identification search results; APEX\manual report review for verification]
-specified site within organ/gross anatomical part (eg within head & neck the site may be specified as

'hypopharynx' or may be defined as 'piriform sinus'; where stated on NHS Lothian Biopsy/Cytopathology Request Form, may require combination of Nature of Specimen and Clinical Summary information)[SOURCE: Labs IM&T\coded information from case identification search results; APEX\manual report review for verification/augmentation]
-case type (diagnostic biopsy, cytology, surgical specimen)[SOURCE: Labs IM&T\coded information from case identification search results; APEX\manual report review for verification/augmentation]
-context of tissue removal, stratified by tissue type (diagnostic biopsy & cytology – acute or urgent versus chronic or routine clinical context; surgical specimens – emergency versus elective)[SOURCE: APEX\manual report review; TRAK\EPR\Clinical Notes OR Correspondence for verification/augmentation]
-date of tissue removal [SOURCE: Labs IM&T\coded information from case identification search results]
-age at time of tissue removal [SOURCE: Labs IM&T\coded information from case identification search results]
-sex [SOURCE: Labs IM&T\coded information from case identification search results]
-co-morbidity at time of tissue removal (based on the Charlson or Elixhauser lists, and grouping the defined conditions under broad headings of cardiovascular, respiratory, chronic hepatic, chronic renal, chronic endocrine, chronic inflammatory &/or infection &/or autoimmune condition, malignant diagnosis of any kind within past 5 years, alcohol abuse, smoking) [SOURCE: TRAK\EPR\Clinical History\Previous Coding & TRAK\EPR\Clinical Notes OR Correspondence for verification/augmentation]
-immunosuppression status at time of tissue removal (constitutional or iatrogenic or none) [SOURCE: TRAK\EPR\ECS & TRAK\EPR\Clinical Notes OR Correspondence for verification/augmentation]
-drug history relevant to receptor expression or viral susceptibility (current antiviral for HIV or Hepatitis C, ACE inhibitor, Aldosterone receptor antagonist) [SOURCE: TRAK\EPR\ECS & TRAK\EPR\Clinical Notes OR Correspondence for verification/augmentation]
-date & result (positive or negative) of any SARS-CoV-2 diagnostic test results during the study window [SOURCE: Labs IM&T\coded information retrieved via search using CHI for all cases in the screening cohort]

This data panel will be linked to all cases in the screening cohort by the BioResource, and provided in a pseudoanonymised form to the research team.

Screening strategy
The screening strategy of tissue types has been determined by the hypothetical plausibility of SARS-CoV-2 infection, based on;
i.)published histological evidence of infection of the target tissue type by one or more related betacoronavirus, &/or
ii.)reported clinically relevant features of infection with SARS-CoV-2, &/or
iii.)patterns of SARS-CoV-2 receptor expression in normal human tissue.

Based on current evidence, our screening strategy will therefore stratify cohort testing into the following broad sample groups:
-*Group A*; diagnostic biopsy, lymph nodes and 'small' surgical excision specimens removed from the upper aerodigestive tract (head and neck tissues including nasal cavities, oral cavity, paranasal sinuses, pharynx, middle ear and pharyngotympanic tube; lower respiratory tract including trachea, bronchi, bronchioles and alveoli; mediastinum; upper gastrointestinal tract including oesophagus, stomach and duodenum)
-*Group B;* diagnostic biopsy and surgical excision specimens and cells (in FFPE blocks) derived from body fluids (including body cavity fluids, gastrointestinal tract fluids, cerebrospinal fluid, aspirates of solid organs, glands or

lymph nodes) removed from patients +/- 14 days from the date of a confirmed positive Covid-19 viral swab results.
-*Group C*; diagnostic biopsy and surgical excision specimens from the neuromuscular and central nervous system and cells (in FFPE blocks) from cerebrospinal fluid
-*Group D*; diagnostic biopsy and surgical excision specimens and cells (in FFPE blocks) derived from body fluids not already covered in Groups A, B or C.

RNA extraction and quantitative reverse transcriptase PCR-based detection of SARS-CoV-2 from FFPE tissue samples
The BioResource will provide two pseudoanonymised 10 micron tissue sections (taken consecutively to the section used to create the 'new' H&E) for each FFPE tissue sample in each case within the screening cohort. RNA will be extracted from these sections using an NHS Lothian-validated semi-automated Maxwell® RSC RNA FFPE extraction method (as per the manufacturers instructions). As this project is focussed on sample types not currently used for diagnostic virology testing (FFPE tissue) use of this extraction method will not compete with (foreseeable) clinical demand for processing of diagnostic virology samples.

Concentrations of the extracted RNA will be analysed by spectrophotometry; where initial sections yield less than 1 ng / ul of extracted RNA, further sections will be requested from the BioResource to provide sufficient material for PCR screening. RNA will be screened for the presence of SARS-CoV-2 using an NHS Lothian-validated 'nCoV 2019' quantitative reverse transcriptase PCR assay based on the PHE-endorsed assay currently in use in NHS Lothian diagnostic services (method described in detail in Corman et al, 2020). This test is recommended for use as one of the 'in house' diagnostic assays by the WHO, using the Invitrogen Superscript III one-step rtPCR kit and a primer mix specific to two regions of the RdRp gene. The assay will be run on an ABI7500 (or analogous) analyser, with each run including two negative control FFPE tissue-derived RNA (from historical diagnostic samples taken >12 months prior to emergence of SARS-CoV-2). While this test is not yet validated for use on FFPE tissue, there is precedence and expertise for diagnostic viral testing of FFPE-recovered nucleic acids within NHS Lothian Virology labs, and for molecular testing of FFPE-recovered RNA within NHS Lothian Molecular Pathology labs. We recognise the risk of high demand for reagents for 2019 nCOV testing, and in the event of any shortage owing to clinical needs, alternative PCR-based assays with analogous sensitivity will be sought for this project.

RNAScope™ in situ hybridisation for viral and host RNA in PCR-positive SARS-CoV-2 tissue samples
Following PCR screening, FFPE tissue sections from those cases with detectable SARS-CoV-2 RdRp product will be examined for evidence of viral infection using a highly sensitive and specific modified in situ hybridisation technique (RNAScope™ by Advanced Cell Diagnostics). To date, RNAScope™ probes have been designed to bind specifically to positive-strand viral genomic/subgenomic RNA transcripts (=*antisense* probe) and to negative strand viral RNA intermediaries (=*sense* probe), targeting sequences within the gene encoding the SARS-CoV-2 spike protein. Using both the *antisense* and *sense* probes therefore enables detection (and possibly distinction) of the presence of viral genomic RNA *and* of viral RNA synthesis activity. The spike gene sequences targeted by the probes are highly specific to SARS-CoV-2 and will not detect other coronaviruses. In addition, probes have also been designed to detect host RNA transcripts for relevant infection associated proteins ACE2 and TMPRSS2, and for the crucial antiviral response protein Interferon-beta (IFN-beta).

Consecutive 5 micron sections will be cut onto slides from all cases with PCR-detected RdRp product, with a

series of unstained sections for ISH analysis, and one further H&E-stained section for histological analysis. Sections of historical archived de-identified FFPE tissue taken from patients >12 months prior to the emergence of SARS-CoV-2 will be used as negative control tissue for optimisation of RNAScope™. Unstained sections will be subject to chromogenic ISH assays as per *Advanced Cell Diagnostics* protocols with staining of specific tissue types (epithelial- / connective tissue- / muscle- / neural- / lymphoid-predominant) to be optimised as necessary by varying conditions of deparaffinization, antigen retrieval, permeabilization and incubation conditions.

Confirmation of host factors associated with infection (including ACE2, TMPRSS2 and IFN-beta, assessed with ISH) will also be sought at protein level using antibody detection. Following ISH analysis, the same tissue sections can be stripped and re-stained for chromogen-based immunohistochemical detection of specific proteins, with commercially available antibodies for the SARS-CoV-2 Spike protein, and host proteins ACE2 (surface receptor), CD209L, TMPRSS2, CTSL and CTSB (entry associated proteases), and IFN-beta (all available for use in IHC from Abcam).

The chromogen-stained slides from ISH and IHC assays will be scanned to whole slide images for image analysis, in addition to manual review and scoring by pathologists.

Data analysis of primary aims

Prevalence estimates of SARS-CoV-2 based on PCR screening of cases

As described, some patients may have had more than one 'case' submitted and processed by NHS Lothian during the study window, therefore the total number of screened cases will differ to the total number of patients screened in the study. These 'same patient' cases may reflect sampling of the same organ/gross anatomical part, or may represent different organs/parts. The number of positive PCR results can therefore potentially be expressed as:

-a proportion of the total number of cases (=prevalence of SARS-CoV-2 within the screened cases across the study window),

-grouping cases by patient, a proportion of the total number of patients (=prevalence of SARS-CoV-2 within the screened population across the study window),

-grouping cases by patient and by organ/gross anatomical part, a proportion of the total number of samples per organ/gross anatomical part (=prevalence of SARS-CoV-2 within a specific organ within the screened population across the study window)

As described, the absolute number of FFPE tissue samples within each case may be greater than one, therefore *any* positive PCR result from *any* sample within a single case, or from any sample from the same organ/gross anatomical part from the same patient, will be counted as a 'positive case' (irrespective of whether other tissue samples from the same patient are positive or negative). The number of cases with detectable SARS-CoV-2 RNA will be expressed as a proportion with 95% confidence intervals, adjusted for the estimated sensitivity of the RdRp assay.

Spatial ISH analysis

Analysis of ISH outputs will entail i.) classification of cells into three viral infection 'states': absence of antisense signal & sense signal (=*no* viral infection); presence of antisense signal / absence of sense signal (=*extant* viral infection); presence of antisense signal **&** sense signal (=*active* viral infection), and ii.) quantification (=counting) of RNAScope® dots (=single viral or host RNAs), by cell type (eg squamous epithelial / columnar epithelial /

goblet cell / etc) and tissue type (eg epithelium / connective tissue / muscle / nervous tissue / lymphoid tissue). Analysis of IHC outputs will entail classification of cells by presence/absence of target IHC signal. In the first instance, ISH/IHC outputs will be analysed manually in an unselected subset of cases by two independent pathologists, who are blinded to clinical parameters, with all samples separately analysed (if technologically feasible) for non-biased, automated assessment using image analysis computer software (QuPath) using a classifier (optimised per tissue/organ type as necessary) that has been trained by the pathologists. Inclusion of the use of a digital image classifier for automated analysis will require correlation with manual counting outputs with an Spearman rank correlation of >0.9. Estimated viral load per positive sample is calculated by extrapolation of Ct value from the initial screening PCR result to standard curves described in Corrman et al.

Infection state (no / extant / active viral infection) will be correlated to specific cell-types per tissue, specific tissue-types per organ, and specific cell types per organ, examined using the Chi-squared or Fisher's Exact test. Presence of extant or active viral infection within an organ/gross-anatomical part can be correlated to categorical clinical and demographic factors using the Chi-squared or Fisher's Exact test and to scale demographic factors using the t-test.

The method of quantification of ISH outputs (and the subsequent selection of appropriate statistical test for examination of correlations) will depend on the nature and pattern of signals, and this may differ for different targets/transcripts. A single approach to quantification will be agreed once the nature of signals has been established for each target; the selected approach will be the optimum strategy to enable comparison of outputs across different tissue and organ sites across cases. The possible approaches, and their respective analytical tests are described in Table 1.

	ISH output type	Example data	Example tests
	Ordinal data	Semi-quantitative scoring of signal intensity of spots	Mann-Whitney U test (2 groups) or Kruskal-Wallis test (>2 groups)
	Continuous data	Raw spot counts	T-test (2 groups) or 1-way ANOVA (>2 groups)
	Comparative data	Ratio of score or raw spot count for one probe against another	If comparison of ordinal data Mann-Whitney U test or Kruskal-Wallis test (>2 groups); if comparison of continuous data, t-test or 1-way ANOVA.
	Threshold data	Binary above/below threshold	Chi-squared / Fisher's Exact test (2 groups) or ordinal logistic regression (>2 groups)

Quantitative ISH outputs will be defined per specific cell- and specific tissue-type. The degree of active viral infection can then be compared across cell and tissue type per organ/gross anatomical part, and correlated to the degree of host transcript expression (for ACE2 and TMPRSS2) across all cells per organ/gross-anatomical part, and per specific cell- or tissue-type, and to the tissue viral load (as described).

The data output will consist of representative images of spatial distribution of virus, and graphical presentation of count data of virus (extant vs active) and virus-host (active infection vs host transcripts) within cell and tissue types.

Conflicts of interest

The authors have no conflicts of interest.

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