

Letter to the Editor

Intracellular residency of *Staphylococcus aureus* within mast cells in nasal polyps: A novel observation

To the Editor:

Chronic rhinosinusitis (CRS) with or without nasal polyps (NPs) (CRSwNP and CRSsNP, respectively) is one of the most common conditions encountered in medicine.¹ CRS is a disease of the mucosa lining the sinonasal cavity characterized by recurrent episodes of inflammation resulting in chronic symptoms such as nasal obstruction, facial pain, rhinorrhea, and reduction in sense of smell.¹ CRS affects up to 15% of the general population in Europe and the United States, ranking it second in prevalence among chronic conditions,¹ and significantly affecting quality of life and health care resources.² Despite the massive expenditure on medical and surgical therapies for this condition, a subset of patients remains resistant to all established treatments.² Identification of either a host or an environmental cause has been unsuccessful. Proposed mechanisms of CRS pathophysiology include the role of superantigens, abnormal cell-mediated immune responses, changes in the inflammatory cytokine cascade, epithelial defects, osteitis of the sinus walls, and viral, bacterial, and fungal factors.¹ Recent guidelines propose the classification of CRSwNP and CRSsNP into 2 distinct pathological entities primarily based on differences in inflammatory cytokine profiles.¹ A CRSwNP T_H2-mediated profile is characterized as eosinophilic with elevated IL-5, IgE, RANTES, and eotaxin,¹ and a CRSsNP T_H1-mediated profile is characterized as neutrophilic with elevated TNF- α , IL-8, and IFN- γ .¹

Emerging evidence implicates bacterial biofilms as mediators of the inflammatory reaction in CRS.¹ Several studies provide strong evidence that bacterial biofilms perpetuate inflammation in CRS and are associated with more severe preoperative disease, persistence of ongoing mucosal inflammation, and poor postsurgical outcomes.³ *Staphylococcus aureus* has been identified as the commonest biofilm-forming microbe in CRS and colonizes the sinonasal cavities in 27% of the patients with CRSsNP and in 60% of the patients with CRSwNP.⁴

Around 20% of the patients with CRS develop NPs,¹ and although studies have clearly identified bacterial biofilms on the sinonasal mucosa of patients with CRS, little data pertain to the bacterial profiles specifically in NPs. We conducted a preliminary study characterizing bacterial profiles in NPs, comparing them with those on nonpolypoidal sinonasal mucosa from the same patients and with non-CRS sinonasal mucosa as control tissue.

A prospective study with full ethical approval was conducted in 9 patients with CRSwNP undergoing functional endoscopic sinus surgery and 5 control patients undergoing transsphenoidal pituitary surgery. Nonpolypoidal sinonasal mucosa and NPs were collected from each patient with CRSwNP and sinonasal mucosa from controls. The bacterial profiles were assessed using fluorescence *in situ* hybridization with confocal laser scanning microscopy (CLSM) and immunohistochemistry. Hybridization conditions were optimized for CRS tissue using appropriate controls (see the [Methods](#) section in this article's Online Repository at www.jacionline.org).

CLSM demonstrated surface-related bacterial biofilms on the nonpolypoidal sinonasal mucosa of all 9 CRS samples ([Fig 1, A](#)),

but not on the epithelial surface of NPs. However, subepithelial and intracellular bacteria were observed in the cytoplasm of host cells in all 9 NP samples ([Fig 1, B](#)). The CLSM Z-axis view indicated that intracellular bacteria were subepithelial in all cases ([Fig 1, C](#)). Bacteria were confirmed within host cells using 4',6-diamidino-2-phenylindole, a nucleic acid stain that resolves the host cell nucleus, surrounded by densely packed bacteria filling the cytoplasm ([Fig 1, D](#)). Biofilms were not observed in any control samples. Species-specific fluorescence *in situ* hybridization identified brightly fluorescent bacteria in CRS samples including *S aureus* in 78%, *Haemophilus influenza* in 33%, and *Pseudomonas aeruginosa* in 33% of the patients (see [Table E1](#) in this article's Online Repository at www.jacionline.org). Immunohistochemistry confirmed our finding of subepithelial intracellular *S aureus*, using an *S aureus* mAb ([Fig 1, E](#)). Immunohistochemical colocalization on sequential sections identified the *S aureus*-harboring host cells as mast cells (MCs) ([Fig 2, A-D](#)).

Our study directly compared nonpolypoidal sinonasal mucosa with NPs from the same patient (thus controlling for host genetics) and is the first to observe both subepithelial bacteria in *ex vivo* NP tissue and intracellular localization of *S aureus* within MCs. This contrasts with recent studies that have shown intracellular *S aureus* in nonpolypoidal CRS sinonasal mucosa.⁵ Sachse et al⁶ demonstrated intracellular *S aureus* replication *in vitro* in NP epithelial cells by introducing an *S aureus* strain to a cell culture model. The mechanism of bacterial migration from surface-related biofilms to intracellular reservoirs remains unclear. However, growing evidence suggests that alterations in the epithelial layer compromise its function as a defensive barrier against environmental allergens and pathogens, as well as a mediator and regulator of innate and adaptive immunity.⁷ A reduction in tight junction proteins and increased proinflammatory cytokines IFN- γ and IL-4 within NPs lead to a disruption in epithelial integrity, induction of epithelial remodeling, and the formation of surface defects.⁷ A defective epithelial layer may provide an opportunity for surface-related bacterial biofilms, already present in CRS, to disperse and penetrate into the tissue. This may explain the absence of surface-related biofilms observed on NP tissue in our study compared with those seen on sinonasal mucosa.

Initially, the bacteria-harboring host cells were presumed to be mononuclear phagocytes, but our immunohistochemical findings rather unexpectedly confirmed them to be MCs. This constitutes a novel finding in NPs. MCs appear to play an important role in promoting innate immunity against microbial pathogens.⁸ Through activation of CD8⁺ T cells, MCs possess the ability to regulate both the T_H1 and T_H2 cytokine pathways, and can therefore adopt both immunosuppressive and immune-stimulatory properties.⁸ Recently, MCs have been demonstrated to exert phagocytosis-independent antimicrobial activity against *S aureus*, mediated through extracellular traps and the release of antibacterial enzymes.⁹ *S aureus* has been shown *in vitro* to subvert these extracellular antimicrobial mechanisms by internalizing within MCs.⁹ Once within the MCs, *S aureus* appears to access the nutrient-rich cytosol and upregulate cell wall synthesis, allowing persistent and viable intracellular SA reservoirs to be established.⁹

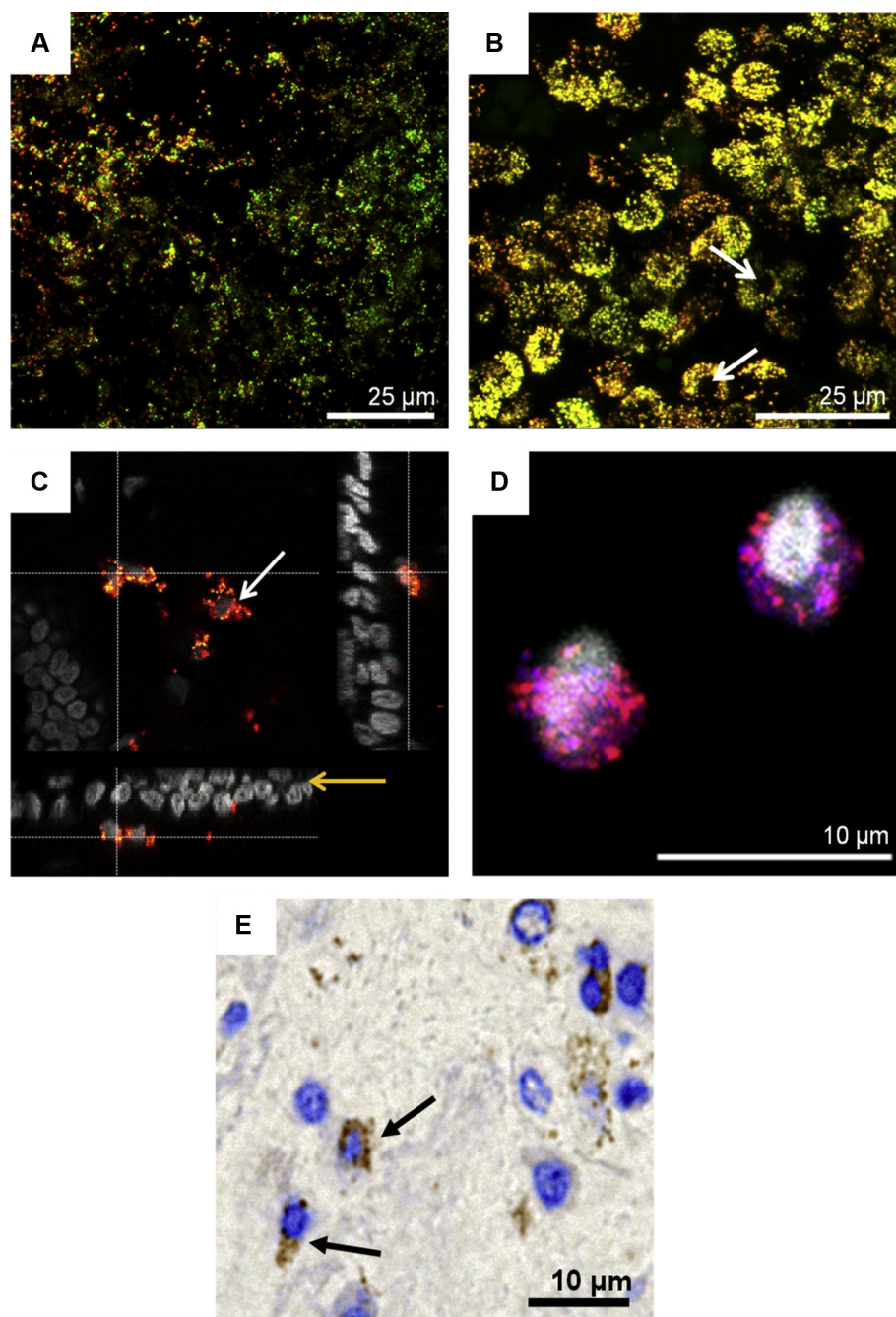


FIG 1. Intracellular *S aureus* in *ex vivo* NPs. Representative CLSM images of sinus mucosa and NP tissue following FISH showing *Staphylococcus aureus* (yellow), *Staphylococcus* genus biofilms (red), and bacteria hybridized only with a eubacterial probe (green). **A**, Aggregated bacteria are widely distributed over the epithelial surface of sinus mucosa. **B**, In contrast, bacterial reservoirs surround host cell nuclei in NP biopsies (white arrows). **C**, XYZ view: staphylococcal aggregates (white arrow) clearly visible beneath the epithelial surface (yellow arrow). **D**, Intracellular *S aureus* reservoirs (pink) colocalized with DAPI-stained host nuclei (gray). **E**, Immunohistochemistry staining with mouse anti-*S aureus* (brown) in an NP biopsy section demonstrating *S aureus* within the cytoplasm of host cells (black arrows) ($\times 40$ magnification). DAPI, 4'-6-Diamidino-2-phenylindole, dihydrochloride; FISH, fluorescence *in situ* hybridization.

The MC in the context of chronic *S aureus* infection may well act as a double-edged sword.⁹ Although promoting innate immunity against microbial pathogens, the MC may be providing a safe haven for *S aureus* by protecting it from extracellular antimicrobial compounds. This not only avoids clearance but also facilitates the establishment of an intracellular microbial reservoir

that could lead to persistence and chronic carriage. This may explain the high levels of resistance to systemic antibacterial therapies in chronic conditions such as CRS. Crucially, these intracellular *S aureus* reservoirs may constitute potential future therapeutic targets for the development of novel bacterial eradication strategies, aimed at reducing systemic antimicrobial usage,

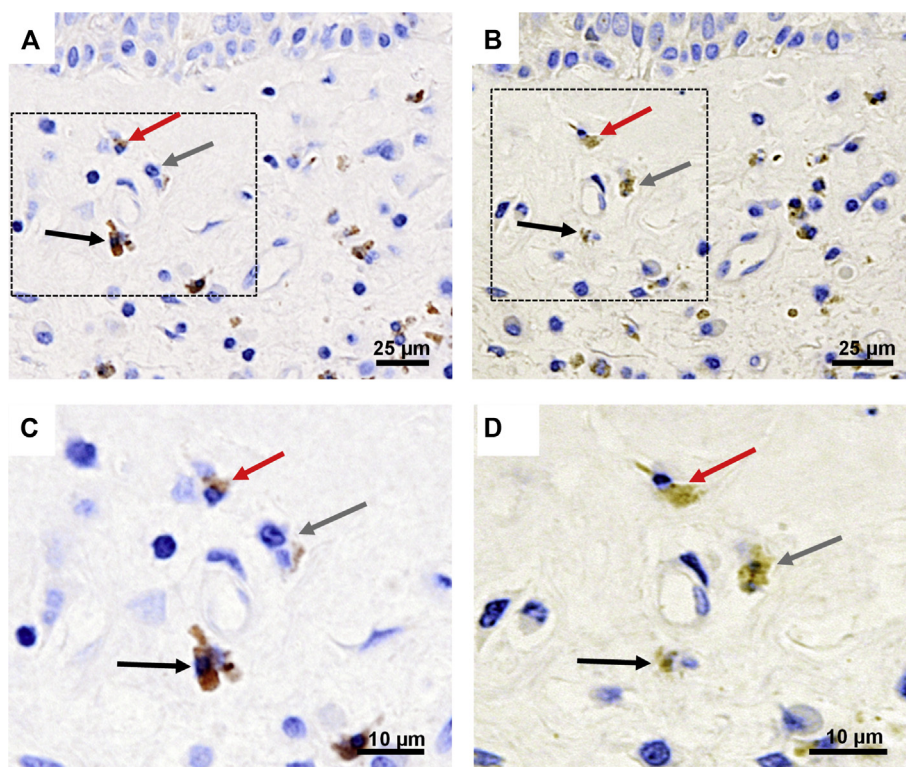


FIG 2. Immunohistochemical colocalization. Photomicrographs of sequential 2- μ m sections of NP tissue stained with anti-AA1 MC tryptase (**A**) and mouse anti-*S aureus* (**B**) demonstrating subepithelial intracellular *S aureus* within MCs (arrows) ($\times 20$ magnification). Both images are shown at higher magnification (**C** and **D** $\times 40$ magnification).

and in turn, the associated risk of antimicrobial drug resistance. To clarify mechanisms underlying survival of *S aureus*, mechanistic studies using *in vitro* MC culture models are currently being planned.

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METHODS

Subjects

Nine consecutive patients with CRS with NPs, meeting the diagnostic criteria defined in the latest European position paper on rhinosinusitis and nasal polyps 2012,^{E1} were included in the study. These patients underwent functional endoscopic sinus surgery by the senior authors (P.G.H. and R.J.S.) at University Hospital Southampton NHS Foundation Trust after having failed an 8-week trial of maximal medical therapy. None of the 9 patients had used either topical or systemic corticosteroid, antibacterial, or antihistamine therapies in the 8 weeks before surgery. Exclusion criteria included age less than 18 years and patients suffering from cystic fibrosis or primary ciliary dyskinesia. All subjects were skin-prick allergy tested to a standard battery of 10 common aero-allergens (ALK-Abelló, Reading, Berkshire, United Kingdom) before surgery. Radiological severity of sinus disease was graded on a computed tomography sinuses scan using the Lund-Mackay scoring system.^{E2} Preoperative demographic data were collected including age, sex, atopic status, previous sinonasal surgery, medical history, history of asthma, history of aspirin sensitivity, and smoking habits (Table E2).

Control tissue was obtained from 5 patients with no clinical history or radiological evidence of CRS undergoing endoscopic trans-sphenoidal pituitary surgery at the Wessex Neurological Centre, University Hospital Southampton NHS Foundation Trust.

Ethical approval was obtained from the United Kingdom NHS Research Ethics Committee (REC 09/H0501/74). All participants within the study provided informed written consent before inclusion in the study.

Tissue specimens

Operative mucosal specimens and NP tissue were obtained from each patient with CRS. Matching mucosal samples were obtained from each control. A swab was taken from the middle meatus and submitted for microbiological analysis. Tissue specimens were placed in HBSS (Gibco, Paisley, United Kingdom) and transported on ice for orientation.

Each sample was gently rinsed in PBS twice for 5 minutes to remove any planktonic bacteria before fixing or analysis. This was performed because planktonic bacteria (including normal nasal flora microbes) were identified on the surface of CRS and control tissue in unrinsed samples when tested with BacLight LIVE/DEAD viability nucleic acid probes. This is consistent with findings in the literature.^{E3} In our study samples, gentle rinsing of the tissue twice before analysis appeared to remove planktonic bacteria but did not disrupt the bacterial biofilms.

Fluorescence *in situ* hybridization

The protocol was adopted from Nistico et al.,^{E4} optimized for CRS tissue. Briefly, specimens were placed in 4% paraformaldehyde in PBS followed by serial washes with PBS and finally stored at -20°C in PBS-ethanol (1:1). The 16S rRNA fluorescence *in situ* hybridization (FISH) probes used were as follows: *Staphylococcus aureus* (Sau) 5'-GAAGCAAGCTTCTCGTCCG-3', *Staphylococcus* genus (Sta) 5'-TCCTCCATATCTCTGCGC-3', *Haemophilus influenza* (Hinf) 5'-CCGCACTTTCATCTCCG-3', *Pseudomonas aeruginosa* (PaerA) 5'-GGTAACCGTCCCCCTTGC-3', and the universal eubacterial consensus sequence probe (Eub338) 5'-GCTGCCTCCGCTAG-GAGT-3'. A maximum of 3 probes were used on each specimen to limit cross-interference with the 3 labeled fluorescent dyes, Cy3, Cy5, and 6-FAM. Because of limitations of available clinical samples and to facilitate the detection of both Gram-positive and Gram-negative bacteria simultaneously using FISH, we used a single fixation process.^{E4-E6} Samples were incubated with 0.5 mg/mL of lysozyme (Sigma-Aldrich, St Louis, Mo) followed by incubation with the probes mixed with hybridization buffer, containing 1 mol Tris HCl, 5 mol NaCl, 20% formamide, and 10% SDS, for 2 hours at 48°C . Finally, samples were submerged in wash buffer at 46°C for 15 minutes, followed by immersion in PBS and visualized using CLSM. In this study, the hybridization conditions including formamide concentration for all the 16S rRNA probes were optimized using common respiratory bacterial isolates and using tissue samples as described in previous publications.^{E4-E6} Briefly, fluorescently conjugated 16S rRNA probes were applied to fixed,

permeabilized bacterial cells under stringent hybridization conditions for each probe. Stringency conditions were achieved by changing the formamide concentration incrementally by 5% from 0% to 50% together with the salt concentrations, while keeping hybridization and wash temperatures constant. Optimal stringency was empirically determined to be the highest formamide concentration not resulting in loss of fluorescent intensity on the target pure culture bacterial representative species. Nonspecific hybridization of oligonucleotide probes was not observed with any clinical isolates of *Streptococcus pneumoniae*, *H influenza*, *Moraxella catarrhalis*, *P aeruginosa*, and *S aureus* tested. All isolates hybridized with the eubacterial 16S rRNA probe (Table E2).

Confocal laser scanning microscopy

CLSM imaging was performed as previously described.^{E5} Briefly, after staining, tissues were mounted (IBIDI) within chamber slides with coverslip bottoms and imaged with a Leica TCS SP5 inverted confocal system (Leica Microsystems, Milton Keynes, United Kingdom) using a $63\times$ oil immersion lens. Sequential scanning was used to further eliminate cross-talk interference from multiple fluorophores. Images were collected and analyzed using Leica LAS-AF software.

Immunohistochemistry

The immunohistochemistry protocol was adopted from Britten et al.^{E7} Briefly, immunohistochemistry was applied to glycol methacrylate embedded tissue. Primary antibodies were applied for 20 hours at previously titrated optimal dilutions (Table E3). Appropriate biotinylated secondary antibodies (Dako, Ely, United Kingdom) were then applied for 2 hours followed by application of avidin biotin-peroxidase complexes. Previously titrated chromogen 3,3'-Diaminobenzidine substrate was then applied, and finally the sections were counterstained with Mayer haematoxylin. All experiments included negative control slides with omission of primary antibodies and appropriate isotype-matched antibodies. A positive control model using an isolated CRS *S aureus* strain was used to test the accuracy of the mouse anti-*S aureus* primary antibody (Abcam, Cambridge, United Kingdom) and to optimize the concentration of DAB substrate, before use on study sections. Colocalization was performed using sequential adjacent 2- μm tissue sections immunostained with appropriate comparable primary antibodies.

Biofilm and intracellular bacterial detection rates

For each patient and tissue type (sinonasal mucosa or nasal polyp), 5 specimens were analyzed for bacterial biofilms or intracellular bacteria. Bacteria were defined as being within a biofilm when aggregated in clusters and attached to the sinonasal mucosa.^{E8} The biofilm detection rate represents the number of biofilm positive specimens (α) identified from 5 tissue samples (biofilm detection rate = $\alpha/5$). The intracellular bacteria detection rate represents the number of specimens with observed subepithelial intracellular bacteria (β) identified from 5 tissue samples (intracellular bacteria detection rate = $\beta/5$).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 6.0 software (Graph Pad Software, Inc, San Diego, Calif). Comparisons between clinical groups for FISH, where the mean of the control groups was 0, were made by using the 1-sample *t* test (2 tailed). The level of significance was accepted as a *P* value of less than .05.

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TABLE E1. Biofilm and intracellular bacterial detection rates for NP tissue

Patient no.	Surface biofilms (BDR)	<i>Staphylococcus aureus</i> (IBDR)	<i>Pseudomonas aeruginosa</i> (IBDR)	<i>Haemophilus influenzae</i> (IBDR)	Eubacterial probe
CRSwNP group					
1	0.0	0.6	0.0	0.0	+
2	0.0	0.6	0.0	0.0	+
3	0.0	0.0	0.6	0.2	+
4	0.0	0.8	0.0	0.0	+
5	0.0	1.0	0.4	0.2	+
6	0.0	0.4	0.0	0.0	+
7	0.0	0.0	0.6	0.6	+
8	0.0	0.4	0.0	0.0	+
9	0.0	0.6	0.0	0.0	+
Total	0	7/9 (78%)	3/9 (33%)	3/9 (33%)	
Mean	0.0	0.49	0.18	0.11	
<i>P</i> value	.0	.002*	.086	.139	
95% CI	0.0	0.23-0.34	−0.03 to 0.34	−0.04 to 0.27	
Control group					
1	0.0	0.0	0.0	0.0	−
2	0.0	0.0	0.0	0.0	−
3	0.0	0.0	0.0	0.0	−
4	0.0	0.0	0.0	0.0	−
5	0.0	0.0	0.0	0.0	−
Mean	0.0	0.0	0.0	0.0	

BDR, Biofilm detection rate; IBDR, intracellular bacterial detection rate; +, detected; −, undetected.

**P* < .05.

TABLE E2. Demographics and microbial profile of study population

Patient	Sex (age [y])	SPT	SH	AS	AH	CC	Bacteria detected by FISH				Eubacterial probe	LMS
							<i>Staphylococcus aureus</i>	<i>Staphylococcus genus</i>	<i>Haemophilus influenzae</i>	<i>Pseudomonas aeruginosa</i>		
CRSwNP												
1	F (48)	—	+	—	—	—	+	+	—	—	+	10
2	F (20)	—	—	—	—	—	+	+	—	—	+	13
3	M (64)	—	+	—	—	+ C	—	—	+	+	+	9
4	F (21)	—	—	—	—	—	+	+	—	—	+	15
5	F (24)	—	+	—	—	+ CNS	+	+	+	+	+	22
6	F (56)	+ G	+	—	+	+ C	+	+	—	—	+	19
7	F (73)	—	—	—	—	+ <i>P aeruginosa</i>	—	—	+	+	+	14
8	M (63)	—	—	—	+	—	+	+	—	—	+	15
9	M (40)	—	—	—	—	—	+	+	—	—	+	16
Controls												
1	F (73)	—	—	—	—	—	—	—	—	—	—	0
2	F (76)	—	—	—	—	—	—	—	—	—	—	0
3	M (52)	—	—	—	—	—	—	—	—	—	—	0
4	M (47)	—	—	—	—	—	—	—	—	—	—	0
5	M (45)	—	—	—	—	—	—	—	—	—	—	0

AH, Asthma history; AS, aspirin sensitivity; C, coliforms; CC, conventional culture; CNS, coagulase-negative *Staphylococcus*; F, female; G, grass; LMS, Lund-Mackay Score; M, male; SH, smoking history; SPT, skin prick testing; +, detected; —, undetected.

TABLE E3. Summary of primary and secondary antibodies, sources, species, and working dilutions with DAB as substrate

Primary antibody	Clone	Source	Host species	Working dilution	Secondary antibody	Working dilution
MC tryptase	AA1	Abcam, Cambridge, UK		1:20000		
Neutrophils	NOE	Dako, Ely, UK		1:1000		
Eosinophils	EG2	Life Technologies, Paisely, UK		1:2000		
Macrophages	CD68 PGMI	Dako, Ely, UK	Mouse	1:100	Rabbit antimouse (Dako, Ely, UK)	1:1000
T Lymphocytes	CD3	AbD Serotec, Oxford, UK		1:700		
B Lymphocytes	CD20	Dako, Ely, UK		1:2000		
Epithelial cells	PanCK	Sigma-Aldrich, St Louis, Mo		1:4000		

DAB, 3,3'-Diaminobenzidine; *UK*, United Kingdom.