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Panel 4: Report of the Microbiology Panel

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Abstract

Objective. To perform a comprehensive review of the literature from July 2011 until June 2015 on the virology and bacteriology of otitis media in children.

Data Sources. PubMed database of the National Library of Medicine.

Review Methods. Two subpanels comprising experts in the virology and bacteriology of otitis media were created. Each panel reviewed the relevant literature in the fields of virology and bacteriology and generated draft reviews. These initial reviews were distributed to all panel members prior to meeting together at the Post-symposium Research Conference of the 18th International Symposium on Recent Advances in Otitis Media, National Harbor, Maryland, in June 2015. A final draft was created, circulated, and approved by all panel members.

Conclusions. Excellent progress has been made in the past 4 years in advancing our understanding of the microbiology of otitis media. Numerous advances were made in basic laboratory studies, in animal models of otitis media, in better understanding the epidemiology of disease, and in clinical practice.

Implications for Practice. (1) Many viruses cause acute otitis media without bacterial coinfection, and such cases do not require antibiotic treatment. (2) When respiratory syncytial virus, metapneumovirus, and influenza virus peak in the community, practitioners can expect to see an increase in clinical otitis media cases. (3) Biomarkers that predict which children with upper respiratory tract infections will develop otitis media may be available in the future. (4) Compounds that target newly identified bacterial virulence determinants may be available as future treatment options for children with otitis media.

Keywords

otitis media, microbiology, virology, bacteriology

Introduction

Otitis media (OM) is caused by viral and/or bacterial infection of the middle ear (ME) space and the resulting host response to infection. Important new work has been published in the areas of microbial pathogenesis, molecular epidemiology, genomics, new viruses, and polymicrobial interactions. This report provides an overview of important recent research in these areas.

Methods

The panel reviewed PubMed to identify important articles related to the microbiology of OM published between July 2011 and June 2015. Keywords included otitis media and *Streptococcus pneumoniae* (*Spn*), nontypeable *Haemophilus influenzae* (NTHi), or *Moraxella catarrhalis* (*Mcat*) or individual respiratory viruses. Members drafted initial reports summarizing advances in their areas of expertise before a composite draft document was circulated to all panel

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members prior to the conference. The panel met at the conference, where the draft was reviewed, additional articles added, and research goals proposed. A final draft was circulated to all panel members for approval following the conference.

Discussion

Virology

Experimental animal/in vitro models. Short et al developed an infant mouse model to study influenza A virus (IAV)- and *Spn*-induced OM.[1] Compared with single challenge, coinfection was associated with greater bacterial burden, inflammatory cell influx into the ME, and concordant hearing loss. IAV-induced inflammation contributed to *Spn* transmission among cohoused naive and *Spn*-colonized mice.[2] H3N2 hemagglutinin facilitated *Spn*-induced OM via activation of proinflammatory mediators.[3] Binding of hemagglutinin to host α2,6-linked sialic acid was negated as a factor promoting viral replication in the ME or secondary bacterial OM.[4] Wren et al[5] showed that transparent colony phenotypes of *Spn* were more adherent than opaque in vitro and that IAV-induced inflammation abrogates this defect in vivo.

It was reported that live attenuated influenza vaccine (LAIV) promotes *Spn* colonization.[6] Mina and coworkers[7] used a murine model to show greater incidence and duration of *Spn*-induced ME disease after live attenuated influenza vaccine inoculation. Tong et al[8] demonstrated that C1qa and factor B deficiencies yielded greater bacterial and viral burdens in IAV and *Spn* coinfected mice and that lacking C5a receptor resulted in fewer *Spn* in the ME regardless of IAV infection.

McGillivary et al[9] identified viperin as a respiratory tract antiviral protein in chinchillas. Transduction of adeno-associated virus encoding *viperin* into the nasopharynx of chinchillas who were then challenged with respiratory syncytial virus (RSV) limited RSV titers. Murrah et al[10] showed that prior infection with adenovirus type 5 resulted in greater proportion of *Spn*-infected ME compared with no virus in the chinchilla. Brockson et al[11] developed a chinchilla model of *Mcat*-induced OM whereby sequential intranasal inoculation of NTHi, *Mcat*, and RSV resulted in culture-positive ME for 11 days.

Epidemiologic, clinical, and viral diagnostics. Peak activity of RSV, metapneumovirus, and influenza A coincided with pediatric office visits for acute OM (AOM).[12] In children with history of recurrent OM undergoing ventilation tube insertion,[13] 71% of ME fluids (MEFs) contained viruses, mostly rhinovirus. In children with acute otorrhea, viruses were detected in 58% of nasopharyngeal samples and 21% of otorrhea samples.[14] In a longitudinal study,[15] viruses were detected in 76% of upper respiratory tract infection (URI) specimens and 27% of asymptomatic specimens. Asymptomatic viral infections were not complicated by AOM. Viral load was higher in URI specimens than in asymptomatic specimens but did not differentiate URI with and without AOM complication.

The clinical relevance of samples positive for rhinovirus RNA was determined by pyrosequencing 179 rhinovirus strains from successive nasopharyngeal specimens from infants.[16] Prolonged presence of rhinovirus >30 days occurred in only 4.5%, suggesting that detection of rhinovirus RNA most likely represents an infection within 30 days.

The clinical spectrum of AOM complicating URI was described in 294 children.[17] During days 1 to 7 of URI onset, AOM was documented in 22%. AOM presented at various stages from mild to severe, with perforation.

Ede et al[18] studied lactate dehydrogenase concentrations in nasopharyngeal secretions from children with URI. High lactate dehydrogenase concentrations were associated with AOM, especially during the first 4 days of URI, and with adenovirus, bocavirus, and rhinovirus infections. Christensen et al reported no correlation between AOM severity and C-reactive protein, peripheral blood white blood cell count, or the absolute neutrophil count.[19]

Role of specific viruses. In hospitalized children in Greece, AOM was diagnosed in higher proportions of children with seasonal influenza A than in those with 2009 H1N1 pandemic influenza A,[20] suggesting the lower ability of pandemic virus to induce AOM. In Texas, infants were followed for URI and AOM through a 31-month period before, during, and after the influenza A pandemic.[21] The pandemic virus did not increase the prevalence of URI or AOM but did increase parents' awareness and resulted in more parent-initiated health care visits for respiratory infections.

In Croatia, AOM was diagnosed in 28% of hospitalized children with adenovirus.[22] In Finland, parechovirus was found in 12% of 100 children with AOM[23]; in 4 (33%) children, parechovirus was detected in the MEF with no bacteria.

Primary human bocavirus 1 (HBoV1) infection was associated with symptomatic URI and AOM by serology.[24] Many studies reported human bocavirus (HBoV) detection in the nasopharynx and/or MEF of children with OM.[25-28] In Taiwan,[25] HBoV was detected in throat swabs from 35 of 705 children with acute respiratory infection, 3 of whom had AOM. In a study of 707 nasopharyngeal samples from 201 children with URI, HBoV1 was detected in 24% and was the only virus in 6%.[28] HBoV1 appeared to be ototropic; 52% of children with URI and HBoV1 had only AOM. In 2 prospective studies, HBoV1 could be detected repeatedly from the nasopharyngeal samples even 3 months apart.[26,28] Because HBoV may have a prolonged presence in the nasopharynx, the clinical significance of HBoV positivity during URI and AOM is still debatable. In children with URI, HBoV1 viral load was not associated with presence of AOM.[26]

In a prospective study, 7% of URI episodes were positive for human metapneumovirus (HMPV).[29] In 3.6% of URI episodes, HMPV was the only virus; of these, 24% were complicated by AOM. HMPV viral load was not associated with AOM.

Polyomavirus was detected in MEFs of children with a history of recurrent OM who underwent ventilation tube insertion.[13] Enterovirus C118 was detected in 2 children with AOM and community-acquired pneumonia.[30]

Viral-bacterial interactions. Van den Bergh et al described distinct bacterial-bacterial, viral-bacterial, and viral-viral associations in 986 nasopharyngeal samples from asymptomatic children.[31] Pettigrew et al reported increased AOM risk with high RSV viral load plus *Spn* or NTHi and with bocavirus and NTHi.[32] Ruohola et al studied nasopharyngeal samples from children with URI/AOM.[33] RSV, without presence of bacteria, was associated with AOM risk. In Australian indigenous children, bacterial loads were higher in children with AOM compared with children without.[34] Children infected with adenovirus were 3 times more likely to have AOM.

In 10 adult volunteers inoculated with rhinovirus, each had a unique bacterial profile; bacterial load did not change during rhinovirus infection.[35] Based on 16SrRNA sequencing, the most abundant genus in the nasopharyngeal samples was *Alloiococcus*, followed by *Corynebacterium, Staphylococcus, Haemophilus, Propionibacterium*, and *Streptococcus*.[36] There was greater diversity of bacterial flora in rhinovirus infected compared with uninfected subjects. *Neisseria* and *Propionibacterium* genera differed significantly between these groups. In South African children with human immunodeficiency virus infection, spectrum of viral URI and bacterial OM were similar to those in uninfected children.[37]

Bacteria: Nontypeable Haemophilus influenzae

Pathogenesis. Hallstrom and coworkers reported the laminin-binding region of protein E was localized to the N-terminus and protein E bound to the heparin-binding C-terminal globular domain of laminin.[38] Lee and coworkers identified signaling pathways critical to development of ME granulation tissue.[39] TGF-β was upregulated in MEs with OM. TGF-β pathway products were higher in NTHi-infected ears than in pneumococci-infected ears. Oh and coworkers studied inner ear inflammation[40] and found that inner ear spiral ligament fibrocytes released CXCL2 in response to NTHi via c-Jun activation, leading to recruitment of polymorphonuclear cells to the cochlea, and that MEK1/ERK2 signaling is required for NTHi-induced CXCL2 upregulation. Preciado and colleagues examined signaling pathways involved with ME metaplasia following transtympanic inoculation of Balb/c mice with NTHi lysates.[41] Microarrays showed that CXCL2 had the largest fold change, with increased expression at 1 and 7 days after NTHi injection.

Harrison and colleagues examined the contribution of Fur in acquisition of iron.[42] Most genes encoding proteins with roles in iron utilization were repressed by Fur. In the chinchilla, Fur was critical for bacterial persistence. Whitby and colleagues described transcription profiles of iron-related genes in vitro and in vivo.[43] The core of iron- and heme-responsive genes consisted of 35 genes maximally expressed under heme restriction and 20 more maximally expressed in heme-replete conditions. Most core modulon members were upregulated in the chinchilla ME during OM.

Harrison and coworkers studied genetic loci important in the stress response.[44] A catalase *hktE* mutant and a peroxiredoxin/glutaredoxin *pgdX hktE* double mutant were more sensitive than the parent to H_2O_2 killing. The *pgdX* mutant was more resistant to H_2O_2 due to increased catalase activity. Binding of iron by Dps mitigated the effect of H_2O_2-mediated killing. An isogenic strain lacking *hktE* and *pdgX* had increased susceptibility to peroxide.[45] These strains had persistence defects in chinchilla OM and in a murine model of chronic obstructive pulmonary disease (COPD).

Raffel and coworkers explored the contribution of Sap to NTHi interaction with the host epithelium.[46] SapA-deficient NTHi demonstrated increased invasion compared with the parent strain. Upon internalization, the *sapA* mutants appeared free in the cytoplasm, whereas the parent strain was found in endosomes, indicating differential subcellular trafficking. Reduced inflammatory cytokines were produced by the epithelium in the *sapA* mutant, and chinchilla MEs challenged with the *sapA* mutant showed decreased disease severity.

Biology of NTHi, biofilms. Pang and coworkers examined the contribution of Dps to NTHi survival in biofilms.[47] A *dps* mutant had a survival defect in high-iron conditions, which was mediated by oxidative stress and restored by genetic complementation. No differences were observed in density and structure of biofilms produced by the parent and *dps* mutant, but survival was decreased in mutant biofilms. Mutant survival was less in chinchilla OM and the mouse pulmonary clearance model.

Jones and coworkers examined the contribution of extracellular DNA (eDNA) to biofilms.[48] Recombinant hBD-3, or (r)hBD-3, bound eDNA in vitro, and eDNA in biofilms in the chinchilla ME colocalized with the chinchilla hBD-3 equivalent. Incubation of (r)hBD-3 with NTHi genomic DNA prevented (r)hBD-3 from inhibiting NTHi biofilm formation in vitro. Establishment of NTHi biofilms in the presence of DNase I and (r)hBD-3 caused a reduction in biofilm height and thickness and rescued the antimicrobial activity of the AMP.

Puig and coworkers studied NTHi from patients with nonbacteremic community-acquired pneumonia, COPD, OM, and invasive disease and from healthy colonized children for ability to form biofilms.[49] Increased biofilm formation was observed for NTHi from patients with invasive disease and OM as compared with NTHi from other patients. Isolates from the oropharynx and MEF had more phosphorylcholine and made denser biofilms than isolates from sputum of patients with COPD or nonbacteremic community-acquired pneumonia. No correlation was found between biofilm formation and the presence of phosphorylcholine in lipooligosaccharide (LOS).

Cho and coworkers examined the role of nuclease.[50] They demonstrated biofilm dispersal in the parent strain, no dispersal in a nuclease mutant, and partial dispersal in a complemented mutant. Microscopic analysis of biofilms

showed increased nucleic acid matrix in the nuclease mutant. The nuclease mutant formed a biofilm in chinchilla OM and demonstrated a propensity to form large aggregates of organisms.

Genetics and genomic studies of NTHi. Using multilocus sequence typing, LaCross and coworkers identified 109 sequence types among 170 commensal and OM-associated isolates from Finland, Israel, and the United States.[51] The largest clonal complex contained 5 sequence types. Little clustering was apparent by disease state or geography. Population structure was evident, with support for 8 populations when all isolates were analyzed.

Zhang and colleagues found the urease operon more prevalent in NTHi causing OM and COPD-associated bronchitis than NTHi from throats of healthy individuals.[52] Strains lacking the operon were much more likely to be from the throat than from OM or COPD isolates.

LaCross and coworkers found 47 hemin receptor (HemR) amino acid sequences among 146 isolates.[53] The predicted structure of HemR was similar to TonB-dependent, ligand-gated channels involved in iron acquisition in other bacteria. Fifteen amino acid polymorphisms were more prevalent among commensal than otitis isolates. After controlling for population structure, only 7 polymorphisms retained significance.

Hariadi and coworkers compared the heme acquisition gene profiles of disease-causing and colonizing NTHi and *Haemophilus haemolyticus.*[54] Four of 5 heme acquisition genes (*hxuA, hxuB, hxuC*, and *hemR*) were more prevalent in ME compared with throat strains. All 5 genes were more prevalent in NTHi than in *H haemolyticus*.

Interactions with the host immune system. Langereis and colleagues reported increased complement resistance in NTHi from the ME, correlating with decreased binding of IgM.[55] NTHi gene R2866_0112 had a role in complement resistance: gene deletion altered the LOS and increased IgM binding and complement-mediated lysis. In a mouse model, the R2866_0112 mutant was less virulent. The group also reported that NTHi prevented complement-dependent neutrophil-mediated killing: expression of surface oligosaccharides blocked recognition of a critical LOS epitope by replacement with galactose attached to HepIII or through shielding HepIII-β 1,2-Glc by attachment of oligosaccharide chain extensions.[56]

Wang and coworkers examined the role of deubiquitinase cylindromatosis (CYLD) in NTHi-induced inflammation.[57] They reported that in human lung A549 cells and lungs of $Cyld^{-/-}$ mice, CYLD targets the activation of ERK. CYLD also enhanced NTHi-induced upregulation of MAP kinase phosphatase-1, which led to reduced ERK activation and subsequent suppression of IL-8.

Woo and coworkers demonstrated that human ME epithelial cells upregulated DEFB4 (human β-defensin 2) in response to NTHi via NF-κB activation.[58] Deletion of the distal NF-κB binding motif caused reduction in NTHi-induced DEFB4 upregulation. Internalized NTHi existed free in the cytoplasm of epithelial cells after rupturing the surrounding membrane.[59] Human ME epithelial cells inhibited NTHi-induced β-defensin 2 production by NOD2 silencing but augmented it by NOD2 overexpression. NOD2 deficiency reduced inflammatory reactions following intratympanic injection of NTHi and inhibited NTHi clearance from the ME.

Woo and coworkers studied cochlear inflammation in NTHi OM[60] and found that IL-10 receptors were expressed in lateral wall spiral ligament fibrocytes. Rat spiral ligament fibrocyte cells inhibited NTHi-induced upregulation of MCP-1 (monocyte chemotactic protein 1) in response to IL-10. Inhibition was suppressed by silencing IL-10R1 and was mimicked by cobalt protoporphyrin IX and CO-releasing molecule 2. IL-10 suppressed monocyte recruitment through reduction of spiral ligament fibrocyte chemoattractants. IL-10 inhibited NTHi-induced binding of p65 NF-κB to the distal motif in the promoter region of MCP-1/CCL2, resulting in suppression of NF-κB activation.

Streptococcus pneumoniae

Genomics. High-throughput sequencing projects continue to inform our understanding of *Spn* pathogenesis.[61] Small noncoding RNAs were identified in *Spn* through RNA sequencing. Targeted deletions and transposon mutagenesis demonstrated that specific small noncoding RNAs were required for tissue-specific virulence.[62] The genome sequence of a multidrug-resistant serotype 19F OM isolate was recently published[63] and will complement pathogenesis studies.[64] A whole genome sequencing study of 616 *Spn* isolates examined the impact of PCV-7 on *S pneumoniae*. Rates of recombination differed across lineages, and pneumococcal strains appear to evolve by mutation at a consensus rate of 1.0×10^{-6} to 1.5×10^{-6} mutations per base per year. Capsule switches occurred between related lineages, and most preceded introduction of conjugate vaccines. These data indicate that common post-PCV lineages arose by replacement of vaccine serotypes by nonvaccine serotypes that were rare prior to introduction of conjugate vaccines.[65] A whole genome sequencing study of 3085 *Spn* isolates from children identified hotspots for recombination that included genes encoding antibiotic resistance and the PspA and PspC surface antigens.[66]

Mechanisms of Pathogenesis

Biofilms. A proteomic study showed that biofilm bacteria utilize alternative metabolic pathways and downregulate capsule and other virulence factors as compared with planktonic pneumococci.[67] Biofilm pneumococci show an avirulent phenotype and are unable to cause AOM in some animal model systems.[68-70] Marks and coworkers recently showed that changes in the nasopharyngeal environment (including influenza A virus infection) and resulting host changes (including increased temperature, release of ATP and norepinephrine) caused dispersion of nasopharyngeal biofilm bacteria and that the dispersed bacteria ascended the eustachian tube and caused AOM.[70] Dispersed bacteria exhibit different transcriptional profiles than biofilm

bacteria[71]; virulence factors—including capsule, *pspA, ply, pcpA, nanA, nanB*, and bacteriocins—were upregulated, whereas competence genes and adhesins were downregulated. A shift from purine/pyrimidine and amino acid metabolism to carbohydrate metabolism occurred.[71]

In rodent models, *Mcat* was found to increase ascension of pneumococci into the ME, and pneumococci increased the bacterial burden of *Mcat*. Additionally, *Mcat* conferred passive protection against β-lactam killing of pneumococci in dual biofilms.[72]

Biofilm formation and horizontal gene transfer. Recent studies have implicated host GalNAc as a receptor important for biofilm formation[73] and a number of virulence factors, including Ply, CpbA/PspC, and PhpP in biofilm formation.[74-76] Quorum sensing through the LuxS/AI-2 system and competence is required for optimal biofilm formation.[77-79] Additionally, interaction with epithelial cells improves biofilm formation in vitro.[76,80,81] A major portion of pneumococcal biofilm matrix comes from cells lysed by the major autolysin LytA or through fratricide.[81,82] The matrix consists of cellular debris, including DNA and DNA-protein complexes in which LytC appears to be a major component.[83] DNA in the matrix makes the biofilm environment optimal for genetic exchange of fitness and antibiotic resistance genes.[82,84]

Biofilm treatment. Lysins effectively disrupt pneumococcal in vitro biofilms.[85,86] The quorum-sensing inhibitor yd47 prevents biofilm formation in vitro and OM in a guinea pig model.[87] Macrolides and fluoroquinolones are the most effective antibiotics for disrupting biofilms in vitro.[88] The sensitizing milk protein HAMLET increases the potency of antibiotics to both sensitive and resistant pneumococcal strains.[89]

Complement. Phosphoglycerate kinase and elongation factor Tu were shown to be pneumococcal complement regulatory proteins.[90,91] Phosphoglycerate kinase inhibits assembly of the membrane attack complex, and elongation factor Tu binds factor H. In a mouse model of OM, investigators tested the complement-mediated killing of transparent and opaque colony forms.[92] More complement was deposited on the transparent phenotype and was dependent on the alternative pathway. Capsule switching from 6A to 6C was accompanied by a reduction in complement C3 deposition from the donor strain associated with increased virulence in an OM model in the chinchilla.[93] Children with OM were shown to upregulate genes associated with the classical and alternative pathways in peripheral blood monocytes.[94]

Capsule. Regulation of capsule is critical for pneumococcal pathogenesis.[95] A mutation in the gene encoding the arginine transporter ArcD caused a reduction in capsule production and ability to cause OM in a chinchilla model, likely due to differences in the surface association of capsule.[96] A study demonstrated that a laboratory-generated capsular switch led to increased pathogenesis in a chinchilla barotrauma model of AOM.[93] In another barotrauma model of OM, serotype 6C strains showed reduced ability to produce ME disease as compared with serotype 19A isolates.[97] This correlates with the increased ability of 19A isolates to cause AOM and may relate to differences in levels of complement binding.[97,98]

Up to 16% of *Spn* carriage isolates do not encode capsule[66] and strains lacking capsule have been isolated from OM and can cause OM in chinchillas.[99] Nonencapsulated *Spn*, distinct from those to which capsule cannot be assigned, are divided into those that contain a capsule locus but are defective in production and those that lack the locus. This second group is divided into 3 null capsule clades (NCCs): *pspK* positive (NCC1), *pspK* negative but with *aliC* and *aliD* in place of the capsule locus (NCC2), and strains with *aliD* but not *aliC* (NCC3).[100,101] PspK binds secretory IgA, increases adherence to epithelial cells, and enhances colonization in a mouse model.[101,102] All 8 $pspK^+$ isolates tested and half of naturally *pspK*-negative nontypeable isolates caused AOM following transbullar inoculation.[103] When *pspK* was deleted, reduced bacterial burden and decreased pathology were observed. Introduction of *pspK* into the unencapsulated strain R36A enabled this avirulent strain to cause AOM.[103] Classic nonencapsulated lineages may play a role in pneumococcal OM as sources of antibiotic resistance genes.[66,104]

Glycosidases. Glycosidases provide pneumococci with carbohydrates—for example, hyaluronic acid can be degraded and used as a carbon source.[105] Pneumococcal β-galactosidase BgaA binds to host cell surface galactose β,1-4–linked carbohydrates following sialic acid removal by NanA and may represent a new family of adhesins.[106,107] Increased GalNAc residues are present along the eustachian tube and in submucosal serous glands during infection and may serve as receptors for adherence.[73,108]

Mucin production can be synergistically increased in the presence of pneumococci and NTHi.[109-111] How pneumococci reach the epithelial cell surface through the mucins is unclear. Production of a truncated and secreted zinc metalloproteinase by some strains allows cleavage of MUC16 from epithelial cells, penetration of the glycocalyx, and increased adherence.[112,113]

Additional pathogenesis papers. Cyclic-di-AMP regulates complex cellular processes that can be degraded by phosphodiesterases.[114] Cron et al identified 2 pneumococcal cyclic-di-AMP phosphodiesterases (Pde1 and Pde2) that contribute to OM in a murine model.[115,116] Single mutants in PspA, CbpA, and pneumolysin were significantly reduced in bacterial counts in the chinchilla OM model, although the CbpA mutant was only mildly attenuated.[117,118] A double PspA/CbpA mutant was reduced in pathogenicity similar to the PspA mutant, but a PspA/pneumolysin double mutant showed no reduction in pathogenicity.

Polymicrobial interactions. Profiling studies in children based on 16S rRNA highlight the association among the respiratory microbiota, *Spn* colonization, and OM susceptibility.[119-

[121] Taxa, including *Staphylococcus*, have been negatively associated with colonization by *Spn.*[120] *Corynebacterium* and *Dolosigranulum* have been shown to be protective for development of OM.[121] *Actinomyces, Rothia, Neisseria*, and *Veillonella* were associated with increased odds of AOM.[121]

Moraxella catarrhalis

Genomics of Mcat. Davie and colleagues compared 12 genomic sequences[122] and observed a surprising degree of gene conservation; in addition to conserved core genes, a set of >600 unevenly distributed gene clusters was observed, and there was a low rate of mobile genetic elements.[123] A genomic sequence was released for *Mcat* BBH18, an isolate from a COPD patient,[124,125] and it had 10 unique open reading frames (ORFs), including 8 in a contiguous region of the genome.

Epigenetic variation. Phase-variable DNA methyltransferases (modM) were described in *Mcat*, and differential methylation was demonstrated in accordance with phase status of mutant strains.[126] Analysis *Mcat* isolates from carriage and disease revealed 3 modM alleles (modM1, modM2, and modM3); modM3 was disproportionately represented in the OM isolates.

Transcript profiling. Hansen's group constructed a microarray based on *Mcat* ATCC 43617 to perform transcript profiling. Hoopman et al studied exposure to oxidant and observed increased expression for oxyR, catalase (*katA*), and peroxidase (*ahpCF*), with results validated by real-time polymerase chain reaction and confirmation of oxidant sensitivity phenotypes for isogenic mutants.[127]

The same group used microarrays to study colonization of the chinchilla nasopharynx.[128] Growth in vivo demonstrated >100 gene products with increased expression and >200 with decreased expression: increased expression for at least 5 regulatory genes and increased expression of nitrate/nitrite metabolism genes, most notably aniA, with the latter suggesting that the *Mcat* population is in a biofilm. Several surface factors associated with adherence (hag, mcaP, and mchA1) were decreased in the colonizing population. An *Mcat* isogenic mutant was generated in MC ORF 1550 that was shown to confer a fitness defect in chinchilla nasopharyngeal infections.

Identification of essential genes. Mobegi et al generated a transposon mutant library in *Mcat* BBH18 analyzed by deep sequence analysis to identify ORFs not represented in the viable transposon mutant pools.[129] In sum, 445 potentially essential genes were identified with functions including biosynthesis of fatty acids, vitamins, and isoprenoids. Lead compounds targeting these pathways were shown to have efficacy against *Mcat*, and other OM pathogens.

Metabolism and growth. Genes required for survival of *Mcat* in iron-limiting conditions were identified by a transposon library screen using Tn-seq.[130] Five ORFs were identified in 1 *Mcat* strain as necessary for survival in low-iron conditions. Validation in additional *Mcat* strains revealed that the most significant among multiple strains was yggW, predicted to function in heme acquisition.

Spaniol and colleagues performed transcriptome analyses to find genes with increased expression in low temperature.[131] They observed increased expression of several genes indicating membrane remodeling: an efflux pump, a porin, and other outer membrane proteins. The most notable finding concerned expression of the type IV pilus,[132] which was also increased. This was phenotypically verified as the efficiency of DNA uptake, and transformation was increased at the lower temperature.

Mechanisms of pathogenesis. Using Tn-seq, de Vries and colleagues identified 15 genes that were important to colonization of immortalized epithelial cells,[133] including a glycosyltransferase (lgt1) involved in LOS biosynthesis,[134,135] a putative lipoprotein and an outer membrane protein, entericidin (*ecnAB*), and a regulator with homology to the BadM family. Adherence phenotypes were confirmed with isogenic mutant strains.

Hansen and colleagues used microarray to identify transcripts with increased levels during colonization of immortalized 16HBE14 human bronchial epithelial cells.[136] They identified a lipoprotein (ORF113) with increased expression shown to affect persistence of *Mcat* within the chinchilla nasopharynx.

Buskirk and Lafontaine showed that synthesis of cardiolipin was a determinant of survival of *Mcat* O35E on immortalized epithelial cells, as an isogenic mclS strain lacking cardiolipin had lower recovery from cell infection studies.[137]

Lgt3 is a LOS glucosyltransferase that has been shown to mediate addition of β-(1,4) Glc moieties to the inner core of the LOS.[134] Recently, the function of Lgt3 was shown to be more complex and to include separate transferase domains that mediate addition, respectively of β-(1,3) Glc (domain 1) and β-(1,4) Glc and β-(1,6) Glc (domain 2).[138]

Implications for Clinical Practice and Research Goals

The advances of the past 4 years have several implications for clinical practice (**Table 1**). They also form the basis for the panel's proposed research goals for the microbiology of OM:

1. The role of inflammatory mediators and their mechanisms of action in AOM pathogenesis following viral URI should be studied.
2. The impact of viral and bacterial load in the nasopharynx on generation of local inflammation, AOM development, disease severity, and outcome should be studied.
3. The significance of newly detected viruses, multiple virus infection, and persistent viral infection should be examined.
4. Extend animal models of OM to include common URI viruses, such as rhinovirus.

Table 1. Implications for Clinical Practice.

Research Findings	Implications for Practice
An array of viruses cause URIs. Virtually all URIs can lead to acute and recurrent OM and viral infection alone may cause AOM.	AOM is of viral and/or bacterial etiology. Antibiotic treatment may not be necessary in all cases, while antibiotics alone may be inadequate in others.
Specific ototropic viruses exist such as RSV, adenovirus, and bocavirus.	This confirms a need for specific viral vaccines for prevention of OM.
Peak activities of RSV, hMPV, and influenza coincide with AOM office visits.	Practitioners need to anticipate increased AOM visits during these virus seasons.
During the first 7 days of URI, children may manifest a wide range of tympanic membrane inflammation and a wide spectrum of AOM.	This information should help guide practitioners in AOM diagnosis.
A specific biomarker, LDH, is associated with the presence of AOM during the first four days of URI.	The likelihood of AOM during URI might be predicted using specific biomarkers. Future research in this area should provide more guidance.
New virulence determinants central to the molecular pathogenesis of OM have been identified for *Spn*, NTHi, and *Mcat*.	Compounds that target these determinants and the pathways they control might provide new OM treatment options in the future.

Abbreviations: AOM, acute otitis media; HMPV, human metapneumovirus; LDH, lactate dehydrogenase; *Mcat, Moraxella catarrhalis*; NTHi, nontypeable *Haemophilus influenzae*; OM, otitis media; RSV, respiratory syncytial virus; Spn, *Streptococcus pneumoniae*; URI, upper respiratory tract infection.

5. Evaluate whether specific viruses interact with specific bacteria and elucidate mechanisms of viral-bacterial interaction on the mucosal level.
6. Understand how OM pathogens interact with one another and with commensals in the nasopharynx and ME.
7. Prevention of AOM by prevention and/or early treatment of viral URI should be studied.
8. Understand host genetics in URI susceptibility and AOM development following URI.
9. Additional studies to longitudinally examine the microbiota in the nasopharynx and ME.
10. Exploit advances in bacterial genomics to understand mechanisms of pathogenesis, molecular epidemiology, and emerging antimicrobial resistance patterns.
11. Develop therapeutics effective against biofilms by further studying their role in pathogenesis.
12. Understand how OM pathogens interact with one another and with commensals in the nasopharynx and ME.
13. Better understand the pathogenesis of nontypeable *Spn* OM.

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