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Potential for breath test diagnosis of urease positive pathogens in lung infections

William R Bishai[1] **and Graham S Timmins**[2]

[1] Department of Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland, United States of America
[2] Department of Pharmaceutical Sciences, UNM College of Pharmacy, Albuquerque, New Mexico, United States of America

E-mail: gtimmins@salud.unm.edu

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Abstract

Roles for urease in virulence are accepted for *Helicobacter pylori* and urinary tract pathogens. However, urease is widely expressed by bacterial and fungal lung pathogens causing emerging and opportunistic lung infections, organisms causing acute exacerbations of chronic bronchitis, mycobacterial lung diseases such as tuberculosis (TB), and ventilator associated pneumonia and health care associated pneumonia. Detection of urease provides a method for rapid *in vivo* detection of these lung pathogens by inhaled ^{13}C- breath test, and this review discusses the range of lung pathogens that might be amenable to rapid diagnosis.

Introduction

Urease allows a range of microbial pathogens to hydrolyze urea, a product that is ubiquitous in the host, to supply available nitrogen for growth, to alter local pH or to provide ammonia to cause local tissue damage for invasion. Although urease is recognized as a virulence factor in urinary tract pathogens and *Helicobacter pylori*, it is expressed by a much wider range of pathogens, especially respiratory pathogens in which there is broad variation in urease enzymology, mechanisms of urease regulation, and roles of urease in virulence. These variations, especially in urease regulation, have only recently become fully appreciated, and have implications for the use of urease for *in vitro* and *in vivo* diagnosis of lung infections. Here, we review what is known about urease, its detection *in vitro* and *in situ*, and its regulation and role in virulence for these respiratory pathogens, focusing upon emerging bacterial and fungal respiratory pathogens, mycobacteria, and pathogens causing serious pneumonias (health care associated and ventilator associated pneumonias, hospital acquired pneumonia (HAP) and VAP, respectively).

Urea is a freely available nutrient in lung

Urea, the major urinary excretory waste product of nitrogen metabolism, is present in human plasma at between 2.5 and 8 mM [1]. However, widespread expression of extra-renal urea transporters in most tissues [2] leads to its distribution throughout the body at levels similar to those of plasma. In lung airway surface fluid (ASF) urea is 2–4 mM [3], and in rapid equilibrium with plasma: for example, increasing the dwell time of bronchoalveolar lavage fluid for just 1 min increased the urea recovery by almost 200% indicating rapid exchange of plasma urea into lung fluids. Conversely, transport of from lung ASF into plasma is just as rapid [4]. Thus, by providing a nitrogen source at 2–4 mM, urea could be an important potential nutrient for urease expressing microbes in the lung, with any microbial depletion of urea in ASF being rapidly replaced from the plasma. Alternatively, a major mechanism of microbial control in the lung is their phagocytosis into and killing in acidified phagosomes and phagolysosomes, and so the ability to utilize lung urea to provide neutralizing ammonia may also be important in enabling microbial survival and eventual infection.

Urease K_m optimization and regulation

Ureases hydrolyze urea into ammonia and carbon dioxide. The enzymology and general genetics of microbial ureases have been well reviewed, as have their potential virulence roles, and a number of

Table 1. Urease expression regulatory systems, michaelis constants (K_m) for selected lung pathogens and *H. pylori*.

Pathogen	Urease K_m (mM)	Urease expression control
S. aureus		pH, Rot, biofilm. Agr?
K. pneumonia	0.28 [13]	nitrogen
P. aeruginosa		Nitrogen/rpoN
M. morganii	0.24 [12]–0.7 [14]	pH (direct enzyme activation)
Proteus spp.	10 [12] to 60 [14]	Urea/UreR
M. tuberculosis	0.3 [15]	Nitrogen
U. urealyticum	2.5 [16]	Constitutive ?
H. pylori	0.3 [17] to 0.8 [18]	Constitutive, Nickel

excellent reviews and perspectives are highly recommended [5–10]. The Michaelis constant (K_m) of a pathogen's urease is usually associated with locally available urea levels at its typical site of infection. For example the urease of *Helicobacter pylori*, a pathogen of the stomach where urea concentrations are relatively low, has a K_m of 0.5 mM [11]. In contrast, the urease of *Proteus mirabilis*, a pathogen of the urinary tract where urea is abundant (50–150 mM) has a K_m of 10 mM [12]. Although the reasons for the correlation between local urea concentrations and K_m are uncertain, most ureases are strictly intracellular, and most urea transporters of pathogenic microbes are passive and not active. Thus a urease K_m appreciably lower than extracellular urea concentrations enables metabolism of intracellular urea concentrations down to low levels (at or below the K_m) to drive passive influx of urea along its concentration gradient. This relationship also holds for lung pathogens as ASF urea levels of 2–4 mM [3] are significantly higher than the K_m of most lung pathogen ureases that are known (table 1). Different species of microbes utilize three major mechanisms to regulate urease expression: control by available nitrogen, direct upregulation by urea, and upregulation by acidic conditions, although some species-specific alternate mechanisms occur.

Detection of microbial urease *in vitro* by conventional culture

The most common urease assays are phenotypic assays that asses an isolates' ability to hydrolyze urea in a weakly acidic medium, thereby causing a pH indicator color change. The different urease phenotypic media, developed many years ago, often contain peptones or other nitrogen sources at significant levels, e.g. Christensen's urea agar contains 1 g l^{-1} peptone providing roughly 10 mM of freely available nitrogen. However, it has become apparent in recent years that the regulation mechanisms of urease expression are quite varied between organisms. As shown in table 1, it can be upregulated through general nitrogen limitation (e.g. *P. aeruginosa, M. tuberculosis*) directly by available urea concentrations (*Proteus mirabilis*) or by acidic pH (e.g. *S. aureus*), in *M. tuberculosis* maximal urease activity was only observed at less than 1 mM of available nitrogen [15]. Therefore, using a nitrogen rich urease media such as Christensen's urea agar might result in less than maximal urease expression in organisms whose urease expression is controlled through nitrogen availability. This phenomenon has been shown experimentally for *Acinetobacter anitratus* a species highly related to *A. baumannii* and also known to cause respiratory infection [19, 20], with urease negativity on Christensen's and other peptone or tryptone containing media that was not observed in media without these additions [21]. Even in the case of urease media that contain very low non-urea nitrogen (typically 0.1 g l^{-1} yeast extract), if the inoculum was prepared in nitrogen rich media it may take some time for full urease expression to occur in nitrogen-regulated organisms. This may account for some of the variability in urease positivity occasionally seen in the literature, especially in organisms whose urease expression is tightly regulated by available nitrogen. This is an area that should receive more study, as it is likely that some misinterpretations of urease phenotypic negativity exist.

Detection of microbial urease *in vivo* in lung by breath test

Another issue for conventional phenotypic tests is that they require the acquisition of a representative sample from the infection site, and usually further growth and isolation of the sample: this may be difficult and time-consuming, and also require invasive procedures such as bronco-alveolar lavage. Therefore, *in vivo* breath tests have been developed in which isotopically labeled urea is administered to a specific anatomical site, and the elimination of urease-derived labeled CO_2 in the breath measured. The first developed application involves a labeled urea drink to detect urease positive *H. pylori* in the stomach, with several clinical products available (e.g. BreathTek® by Otsuka, BreathID® by Exalenz). We have been developing an inhaled ^{13}C-urea breath test to detect urease positive pathogens in the lung, have shown preclinical proof of concept in a rabbit model of TB [22] and have extended the approach to other chemistries [23–25]. We recently reported a clinical trial of orally administered urea breath test in TB showed acceptable sensitivity, but with a low selectivity due to a wide range of GI tract urease positive organisms [26].

We published the first clinical data of the inhaled ^{13}C-urea breath test in controls and in cystic fibrosis (CF) patients with *P. aeruginosa* colonization [27]. The increase in exhaled $^{13}CO_2$ after inhalation of nebulized ^{13}C-urea was dependent both upon *P. aeruginosa* colonization status and upon ^{13}C-urea dose. This increase was also very rapid, and maximal at the earliest point

measured, 5 min after nebulization, so that potential *H. pylori* GI signals may not be present at these early time points, although suppression of *H. pylori* urease could also be performed by oral bismuth salt therapy if necessary [28]. Thus, it would appear that microbial urease activity can be readily determined *in vivo* in the lungs of patients. The clinical utility of the inhaled urea breath test remains to be determined, but upon appropriate validation might enable rapid and early detection of lung infections and their response to antibiotics.

Urease as a broad diagnostic target in lung infections

Since initially proposing that the lung pathogens, *M. tuberculosis* and *P. aeruginosa* can be detected *in vivo* by inhalation of ^{13}C urea and assay of $^{13}CO_2$ in breath, we realized that a much wide range of lung pathogens express urease. These are not limited to bacteria, but also include important fungal lung pathogens [29–31] , and we review the available literature upon urease in lung pathogen metabolism and virulence at the species level. First, we cover fungi and atypical or emerging lung pathogens such as *Ureaplasma urealyticum* and *Cryptococcus neoformans* in which clear virulence roles are established. Next we cover the intermediate virulence case of Mycobacteria. Finally, we cover bacterial pathogens causing ventilator associated pneumonia (VAP) and HAP in which the role of urease in virulence appears weak to non-existent, but that may enable diagnosis by breath test.

Species specific role of urease in virulence and diagnosis in lung infections

Opportunistic and emerging lung pathogens

A range of less frequently observed and opportunistic pathogens of the lung express urease, and frequently exhibit interesting properties: for example, *Ureaplasma urealyticum* derives most of its ATP from urea hydrolysis, while major roles for urease have been demonstrated for urease in the virulence of some fungal pathogens.

Cryptococcus, coccidioides and aspergillus fungi

Although several pathogenic fungi express urease, the field is best developed *in C. neoformans*. Urease is overwhelmingly expressed, for example in *C. neoformans* isolates 285/286 showed rapid urease activity [32] and a rapid presumptive test based upon urease activity has been developed [33]. The genetics of the *C. neoformans* urease operon were recently reported, with urease expression both limited by high available nitrogen, and increased by urea [34]. A profound defect in the virulence of a urease mutant of *C. neoformans* was shown in an inhalation mouse model: at day 38 of infection all urease mutant infected mice were alive while all urease positive infected mice were dead [35]. Urease was also powerfully associated with metastasis to the brain after pulmonary infection, through ammonia induced tissue damage enabling microvascular sequestration and brain invasion [34, 36]. Urease is also directly involved in driving a non-protective Type 2 immune response in *C. neoformans* infection [31]. Recently, urease deletion was also shown to significantly alter pathogenesis (time to death) in an intranasal inoculation mouse model of *C. gatii* infection [30], and although related to *C. neoformans*, this pathogen is clearly capable of infecting immunocompetent patients [37]. Cryptococcal urease expression was decreased in mutants in an inositol polyphosphate kinase (IPK), that were also of greatly attenuated virulence in a murine inhaled cryptococcosis, suggesting IPK is important in control of urease expression [38].

In *Coccidioides posadasii*, the causative agent of San Joaqin Valley fever, a similarly profound defect in virulence of a urease mutant was observed in a mouse intranasal challenge model [39]. More recent work showed urease deletion resulted in almost 4 logs less lung CFU of *C. posadasii* in the same mouse model, and a urease deletant showed 60%–70% survival as opposed to 0% survival in the wild type [39, 40]. This group has also shown that a vaccine based upon recombinant urease dramatically decrease lung and spleen burdens, and also increased survival in an intraperitoneal infection mouse model of coccidioidomycosis [41]. Urease was observed to be overexpressed in yeast-phase cells recovered from mouse infection in a transcriptomic study of the related organism *Paracoccidioides brasiliensis* [42].

Aspergillus species cause a range of pulmonary disease including invasive aspergillosis chronic pulmonary aspergillosis, and *Aspergillus* bronchitis in patients with CF, bronchiectasis, lung transplant and artificial ventilation[43, 44]. A study of aspergillosis-causing strains showed urease positivity in 19/20 for *A. flavus*, 15/15 for *A. fumigatus,* 19/30 for *A. niger* and 15/15 for *A. terreus* [45], while isolates from intensive care unit and operating room air were urease positive in 19/20 for *A. flavus,* 15/16 for *A. fumigatus* and 16/19 for *A. niger* [46]. The role of urease activity in virulence is as yet unclear.

Ureaplasma urealyticum

This mycoplasma is an opportunistic pathogen in the premature and neonates and can lead to severe pneumonias [47], with significant lung inflammation and damage [48] and is also linked to bronchopulmonary dysplasia [49]. Hyperammonia syndrome in transplant patients (especially lung transplant) is also linked to this organism [50]. Remarkably, intracellular urease activity generates a significant ammonia chemical potential of 80 mV and proton electrochemical potential of 24 mV, such that 95% of all ATP synthesis is directly dependent upon urease activity [51]. Since

urease is responsible for so much ATP synthesis, urease inhibition might be directly antimicrobial, rather than just modifying virulence. Accordingly, urease inhibition by the proton pump inhibitor lansoprazole [52] lead to ATP depletion and effects on growth, thus demonstrating urease is not just a virulence factor but is essential. Recently, speciation between higher virulence *U. urealyticum* and the lower virulence *U. parvum* has been performed using urease genetic sequence differences and PCR [53], although a correlation between virulence and urease activity was not reported.

Brucella spp.

Lung infection by *Brucella* species is rare, but generally associated with inhalation exposure [54] and has been reported most frequently for *B. melitensis* [55–57]. An unusual *Brucella* isolate with rapid urease positivity caused chronic destructive pneumonia [58] suggestive of a role of ammonia in tissue damage as for cryptococci.

Nocardia spp.

Lung nocardiosis is caused by inhalation of the opportunistic pathogens predominantly *N. asteroids* and *N. brasiliensis*, especially in immunocompromised hosts, COPD [59] and chronic granulomatous disease [60]. Most pathogenic *Nocardia* isolates are urease positive e.g. 29/30 isolates of *N. asteroides* [61], 24/26 of a novel *Nocardia* taxon [62] and 40/40 isolates of *N. farcinica* [63], although some are reported negative which may be due to inappropriate urease media use [64]. There are no reports upon urease genes, enzymology or regulation in these species. The only report on virulence found that most pathogenic strains (mouse foot and intraperitoneal infections) were urease negative [65].

Other opportunistic and emerging urease positive lung pathogens

There are many case reports of other emerging urease positive pathogens in respiratory infections, although nothing is known about the role of urease in these infections. *Rhodococcus equi* causes lung infections mimicking tuberculosis in immunocompromised patients [66–68]. *Ochrobactrum anthropi* can cause pneumonia in a variety of immunocompromised states [69–71]. Similarly, a number of *Sphingobacterium* species isolates from CF patients are urease positive, although their role in lung decline remains unknown [72, 73]. There are a number of reports of respiratory infections by *Corynebacterium pseudodiphtheriticum*, including in immunocompetent patients [74–77]. The opportunistic (predominantly canine) pathogen *Bordetella bronchiseptica* can cause pneumonias in HIV infected patients [78–80]. In *B. bronchiseptica*, urease was not induced by urea or low nitrogen, but temperature shift (30 °C–7 °C) [81]. Recently, the urease operon was detected and sequenced in *Chryseobacterium indologenes*, an emerging drug resistant respiratory pathogen in CF [82] and other immunocompromised states [83]. In fungi, *Exophiala* species are opportunistic respiratory pathogens, and environmental and clinical isolates all expressed urease [84].

Mycobacteria

Most mycobacteria that are pathogenic to either immune compromised or immune competent patients, such as *M. tuberculosis*, *M. kansasii, M. scrofulaceum, M. marinum* and the *M. chelonae-abscessus* complex have long been known to be urease positive at or close to 100%: the exceptions being *M. avium intracellulare complex (MAC)* that is only very rarely positive and *M. bovis* that is probably predominantly positive but has been reported at from 60%–100% due to low numbers of isolates [85–92]. The regulation of *M. tuberculosis* urease has been shown dependent upon overall available nitrogen levels [90, 93] most likely through GlnR [94–97].

A role for urease as a mycobacterial virulence factor has proven difficult to elucidate, the need for a complex operon and the near 100% positivity of clinical isolates of *M. tuberculosis* would argue for its importance in human pathogenesis. Furthermore, the enzymes substrate, urea, can only be obtained from a mammalian host as pathogenic mycobacteria lack the urea-forming degradative enzyme arginase [98]. However, experimental evidence has been ambiguous. Early discussion was based upon the importance of urease derived NH_3 and phagolysosomal arrest [99, 100]. Urease mutants in *M. bovis BCG* were described, and in mouse lung, statistically significant lower CFUs were observed for a urease negative mutant (about 0.5 logs throughout the experimental duration) [101]. However, no survival defect was observed in phorbol ester treated THP-1 cells. Unappreciated perhaps though is the only urea source mentioned (RPMI 1640, 5% fetal calf serum, FCS, and 20 mM glutamine) would have been the 20 fold diluted serum (typically 3–5 mM in urea) [102] leading to about 0.15–0.25 mM urea in the experiment-a value below the urease K_m and quite unlike the physiological condition in which urea concentrations exceed the K_m by 10 fold or more. A similar argument (lack of relevant urea concentration) can be made about macrophage killing results of urease knockout in *M. tuberculosis* as the only urea source was tenfold diluted FCS [93] and although the results of *in vivo* experiments were similar to previously, about 0.5 or so log CFU deficit at many time points throughout lung, liver and spleen, eventually they reached that of the wild-type. More direct studies of phagosomal pH with *M. bovis BCG* in primary mouse macrophages (10% FCS as urea source) did show marked differences in phagosomal pH (7 for wild type, 4.5–5.5 for the urease knockout) [103]. Additional urea was found to

Table 2. Compilation of recent VAP and HAP studies as a function of microbial etiology and potential detectability through urease.

ESKAPE	Urease	NHSN 2009–10 VAP [107] 8474[a]	SENTRY 2004–8 USA VAP [108] 2585[a] (total VAP&HAP)	SENTRY 2004–8 USA HAP (bacterial) [108] 2585[a] (total VAP&HAP)	SPAIN VAP [109] 157[a]	IAP [110] 330[a]
E. faecium	Neg	0.3%	nr[b]	nr	nr	nr
S. aureus	Pos	24.1%	31.9%	36.5%	29.6%	17%
K. pneumoniae	Pos	10.1%	6.6%	8%	6.4%	13%
A. baumannii	Pos	6.6%	5.3%	4.4%	8.9%	1%
P. aeruginosa	Pos	16.6%	21.4%	19%	19.7%	16%
Enterobacter			8.8%	8.6%		13%
E. coli	Neg	5.9%			1.3%	15%
E. faecalis	Neg	0.5%				
M. morganii	Pos				3.8%	4%
Proteus sp.	Pos	1.4%				10%
Serratia sp.	Neg	4.6%	6.5%	5.5%		15%
NON-ESKAPE						
H. influenzae	Pos	nr	12.2%	5.6%	17.4%	16%
Total potentially Detectable by *in vivo* urease		57%	77%	73%	85%	77%

[a] Numbers of infections in study.

[b] Not reported or not reported separately (nr).

potentiate pH dependent and urease-mediated alterations in MHC-II trafficking in phorbol treated THP-1 cells (RPMI with 5% FCS ±2 to 50 mM urea), with a clear urea dose response observed between 0, 2 and 10 mM [104].

Overall, it is difficult to reach a clear conclusion, but urease could be important in aspects of disease not well modeled by mice such as cavitation and/or transmission where urease derived ammonia might cause tissue damage (as has been observed in other microorganisms) [34, 36] and thereby enable dissemination and transmission.

VAP and HAP caused by ESKAPE pathogens and *Haemophilus influenzae*

VAP and HAP are major causes of morbidity and mortality, and are frequently associated with pathogens for which antibiotic resistance is a concern, especially the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) [105]. Although the need for new antibiotics for ESKAPE pathogens is appreciated, drug discovery and development can be slow and complex [106], and rapid diagnosis is often difficult. The extent of known urease expression by the ESKAPE pathogens and the other major pathogen *H. influenza* in several pivotal studies of VAP and HAP, together with a study of Intensive care unit associated pneumonia (IAP) is shown in table 2. It can be seen that urease is expressed by between 60% and 90% of the pathogens causing VAP and HAP in these studies, the major exceptions being the *Enterobacter*. These data hint that should inhaled ^{13}C urea breath test prove clinically effective, it may assist the rapid diagnosis of many cases of VAP and HAP. The characteristics of urease expression, control and role in virulence are therefore discussed for these species individually.

Staphylococcus aureus

S. aureus is generally urease positive in clinical isolates e.g. a study of methicillin resistant *S. aureus* (MRSA) in the UK showed 127/129 isolates were positive [111]. Several conditions control urease expression in *S. aureus*. Firstly, urease transcription rapidly increases in response to a shift from neutral to acidic stress, as shown by two microarray studies. In the first, a shift from neutrality to pH 5.5 caused upregulation of urease genes of from 2 to 13 fold [112]. The other study involved a shift to pH 4.5 and showed rapid upregulation of urease genes within 10 min by 6–47 fold [113]. A later study confirmed these findings, also showing that urease was downregulated by alkaline pH [114]. This rapid upregulation of urease could provide defenses when the organism becomes contained in an acidified site, such as after phagocytosis [115]. Other stresses have been shown to upregulate urease. A proteomic analysis showed that the urease beta subunit was increased 11–14 fold treatment with the detergent Triton X-100 [116]. Treatment with antibiotics also appears to upregulate urease. A MRSA strain, COL, increased urease expression upon adaptation to high levels of oxacillin [117] and an increase in urease transcription was also reported during adaptation to daptomycin [118].

Secondly, urease is controlled by several broad metabolic and growth phenotype regulatory pathways. A catabolite control protein A (CcpA) mutant

had downregulated urease gene expression compared to wild type that was also phenotypically observed [119]. The investigators had previously shown RNAIII, a regulatory RNA that is central to the accessory gene regulator (*agr*) response, is decreased in the CcpA mutant [120]. Furthermore, urease was shown to be negatively controlled at transcriptional and enzymatic levels by the *rot* system (repressor of toxins) that appears to act in an opposite manner to *agr* [121]. A *ureF* mutant was also found to decrease the *agr*-regulated alpha hemolysin activity in a transposon screen [122] although the mechanism was not elucidated. A biofilm mode of growth, often associated with antibiotic resistance phenotypes, also increased transcription of urease genes and urease enzyme activity [123]. The biofilm upregulation appears under control of the ClpP protease, as deletion greatly increases expression and enzymatic activity [124] and transcriptional and enzymatic assays also confirmed negative control by rot [121].

It was also shown that the urease operon is expressed in a mouse lung infection model, in levels similar to those seen *in vitro* in stationary phase [125]. Furthermore, in a mouse lung infection model the expression of genes encoding urease subunits and metabolism was higher in USA300 strain than USA400: it was hypothesized this may account for the association of USA300 strains with persistent and recalcitrant infection [126]. Overall, urease regulation in *S. aureus* is consistent with a role in adapting to a range of stresses and growth modes. A role of urease as a classical virulence factor in lung infection has not been reported, but it was an important virulence factor in a mouse model of urinary tract infection [127], a finding recapitulated in kidney infection by Fey and co-workers [128].

Klebsiella pneumonia

K. pneumoniae is urease positive, and capable of growth on urea as sole nitrogen source [129]. Its urease positivity is such that the specificity of oral urea breath testing for *Helicobacter pylori* can be compromised by the presence of *K. pneumoniae* in the stomach [130, 131]. Urease in *K. pneumoniae* is regulated by nitrogen availability through the *ntr* nitrogen regulatory system: although most transcription appears to be regulated through the nitrogen assimilation control protein (NAC) that is under *ntr* control, a second promoter also allows lower levels of NAC-independent transcription [132–135]. When cells were grown in limiting nitrogen, urease expression was increased by 78 fold compared to growth in nitrogen rich conditions [133]. The genome contains the *urtABCDE* operon, an ortholog of a high affinity active urea transport protein that allows urea uptake at very low concentrations in *Synechocystis* [136] and *Corynebacterium glutamicum* [137]. The K_m of this transporter in whole cell *K. pneumoniae* was reported as 13 μM [13], very much lower than the urease K_m of 280 μM [13]. No role of urease in *K. pneumoniae* in virulence in the lung has been shown [138, 139]. However, the initial stage in nosocomial infection that precedes opportunistic infection, is the colonization of the patient's gastrointestinal tract [140]. Urease was shown to be an important virulence factor in GI colonization in a mouse model [141] and a urease mutant was unable to compete for GI colonization with its urease positive parent [142].

Acinetobacter baumannii

A. baumannii is urease positive [143] while other *Acinetobacter* species isolated from humans are also usually positive (97%) [144]. However, results using Christensen's agar have been reported as variable [145] or even with positivity as low as 17% [146] as discussed earlier. Little else has been reported about the urease of *A. baumannii* although it is not required for lung virulence [147]. However in a transposon mutagenesis study, a mutant in the gamma subunit of urease exhibited decreased virulence in a *Caenorhabditis elegans* model [143]. As for *K. pneumoniae*, urease promotes GI colonization in some Acinetobacter spp. [148] although such a role in *A. baumannii* pathogenesis is unclear.

Pseudomonas aeruginosa

Urease expression has long been used in the identification of *P. aeruginosa* [149] and almost all non-CF isolates of *P. aeruginosa* express urease [150]. An ortholog of the pH sensitive *H. pylori* urea transporter gene ureI, encoded by PA3362, is also a predicted cytoplasmic membrane protein and putative urea transporter upregulated by simple amides in media [151] and is part of the *amiEBCRS* operon [152]. Urease regulation by ammonia levels is long known [153] and is upregulated in nitrogen restricted conditions via sensing of intracellular glutamine levels [154, 155] as is urea uptake [156]. Although *rpoN* mutants have defects in virulence [157, 158] many other virulence factors than just urease are modified, and no definitive studies of urease alone are known. Urease is downregulated however, in certain strains and clones with a high degree of adaptation and trophism for CF patients [150, 159]. A recent study of a range of clinical CF and non-CF isolates, however, showed urease activity in all, with relatively little variation and no association with virulence [160]. However, the detection of urease activity in the lungs of CF patients colonized with *P. aeruginosa* [27] shows detectable activity in the lung, even with the potential for downregulation.

Other enterobacteriaceae

Escherichia coli. Most strains do not produce urease due to mutations in the *ureD* gene[161], however some strains do carry an independent plasmid encoded urease gene cluster that is active [162] and positively regulated by urea through a ureR regulation mechanism [163]. *Morganella morganii* is a relatively

infrequent cause of health care associated pneumonia [110]. Its urease has been isolated, and exhibited a K_m of 0.8 mM. No role in lung virulence is known, although it is known that urease protects the organism from low pH in the presence of urea [164]. A lowered cytosolic pH directly activates the urease, which shows a pH maximum of 5.5 (in permeabilized cells) [164] allowing instantaneous protection from low pH as it does not depend upon transcription and translation. *Proteus mirabilis* and other Proteus species are urease positive [130, 165–167] and are relatively rare lung pathogens [168, 169]. The enzyme has been isolated and the K_m for urea reported as 13 mM, higher than many other lung pathogens, but in accord with its usual role as a urinary tract pathogen. Urease is positively and negatively regulated by UreR and H-NS respectively, with both an increase in temperature (25°C–37°C) and presence of urea leading to a loss of H-NS mediated transcriptional repression [170, 171]. Although urease is an established virulence factor in *Proteus* urinary tract infections [172, 173] no roles in virulence in lung infection have been reported.

Haemophilus influenza

H. influenzae causes significant VAP and HAP, and is also causes bacterial induced exacerbations in chronic obstructive pulmonary disease (COPD) [174, 175]. It is urease positive [176] and is expressed in humans during infection, as serum IgG responses against its urease are seen suggesting exposure to urease during infection [177]. Isolates from COPD patient sputum are much more likely to express urease than throat swabs from normal (97% versus 78%) [178] indicating a selective pressure to maintain urease activity in this population. *In* vitro data shows urease is protective against acid stress (pH 4) in a urea-dependent manner [177]. A model of co-infection showed that the *UreB* knockout produced an *in vitro* growth defect, and that *UreH* was required both for single infection with *H. influenza*e and co-infection with Influenza A [179]. A proteomic study showed 8 fold upregulation of the gamma subunit of urease when grown in sputum from COPD patients rather than media, although no control mechanisms were discussed [180]. Although the mechanism of urease regulation is not known at the molecular level, high levels of ammonia caused decreased expression of urease activity, with 50% inhibition by ~75 mM ammonium chloride, and so it is likely that nitrogen availability is at least one regulator. *H. parainfluenzae* is also associated with chronic bronchial disease [181] but appears to have much more variability in urease expression [182].

Potential clinical roles for inhaled urea testing

The rapid and point of care detection of lung infections by using inhaled ^{13}C-urea could prove useful in a number of diseases, especially where conventional sputum samples are either difficult to obtain or are not usually informative. In fungal lung infections, urease positivity could lead to a higher index of suspicion and so lead to more rapid diagnosis of these infections that are often diagnosed relatively late, or that require invasive BAL or CT imaging [183, 184]. In mycobacterial disease, the test could be used to determine the need for further diagnosis, such as GenExpert in TB, or culture for NTMs. The recent ISDA/ATS guidelines on HAP and VAP diagnosis and treatment represent a thoughtful response to the diagnosis and treatment of these diseases [185]. The lack of utility of many current biomarkers such as procalcitonin, Triggering receptor expressed on myeloid cells (TREM-1) or C-reactive protein, led to recommendations to not use these in diagnosis. Furthermore, invasive sampling requirements and slower microbial culture, led to recommendation not to use bronchoalveolar lavage and quantitative cultures in diagnosis. Since many of the concerns around antibiotic stewardship in HAP and VAP are around the potentially resistant and multidrug resistant ESKAPE pathogens, their rapid detection as causative agents in HAP/VAP by inhaled urea breath test might provide further discrimination in antibiotic prescription algorithms and practice, allowing better stewardship. More speculatively, changes in urease activity may prove useful in monitoring antibiotic efficacy, or may predict exacerbation in diseases such as CF. Overall, these potential uses will need validation in a range of clinical trials.

Conclusions

Urea is freely available in lung tissue and ASF for microbial utilization through urease, and a wide range of pathogens capable of causing respiratory infection express urease. In many cases, the role of urease in pathogenesis or virulence is established, but equally, for many organisms it either does not appear important or has not been studied. Roles in production of ammonia for nitrogen utilization and/or local neutralization of acidic pH are established in many cases, but again in many species the role(s) of urease are unclear. The mechanisms of regulation of urease activity are also diverse, and may account for some inconsistencies in reports of urease positivity, depending upon the available nitrogen and pH in the media used. Since most respiratory microbial ureases are inhibited by an FDA approved agent at achievable therapeutic concentrations, and since their activity can now be detected by *in vivo* breath test, including in humans, further studies are indicated to elucidate potential clinical uses of urease detection and inhibition. In the diagnosis of lung infection, the prevalence of urease expression in each individual disease needs to be considered. For example the expression of urease is

less frequent in organisms typically causing community acquired pneumonia (usually only *H. influenzae* at 5%–10%) [186, 187] that for those causing HAP and VAP (60%–90%, table 2), and this will have impacts upon how the test might be used in diagnostic algorithms in different diseases.

Declaration of interests

Dr Timmins is inventor or co-inventor of inhaled ^{13}C-urea technology and patents, these are licensed by STC.UNM to Avisa Pharma, and Dr Timmins benefits from a portion of these license fees. Dr Timmins acts as chief scientific advisor to AvisaPharma, (UNM). Dr Bishai acts as a scientific advisor to AvisaPharma.

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ORCID iDs

Graham S Timmins https://orcid.org/0000-0002-4971-718X

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