Screening Honey for Antibacterial Activity Against *Acinetobacter baumannii*

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1. Introduction

Acinetobacter baumannii is a frequent cause of infections in hospitals around the world, which are difficult to treat due to the organisms inherent antibiotic resistance[1]. It is particularly prevalent in intensive care units (ICUs) with around 18% of *A. baumannii* infections in the UK resulted in bacteraemia (mostly in infants and the elderly) in 2018[2]. Due to the challenge of treating infections caused by this bacteria there is considerable interest in exploring natural products for novel antimicrobial activity[3]. Honey has been used for many centuries in traditional medicines to treat infections. It has antibacterial properties as well as anti-oxidant and anti-inflammatory activities.

The aim of this project is to screen our collection of 270 honey samples for the presence of natural compounds, which demonstrate antibacterial activity against clinical isolates of A. *baumannii.*

Hypothesis: Honey samples from geographically distinct locations may contain antibacterial compounds with activity against *A. baumannii*

Objectives

1. To confirm the identity of clinical isolates of *A. baumannii* provided by Public Health Wales using phenotypic and genotypic methods.
2. To determine sensitivity to antibiotics commonly used to treat this pathogen.
3. To determine sensitivity of isolates to honey
4. To identify honey samples worthy of future study as a potential source of antimicrobial compounds with activity against *A. baumannii*

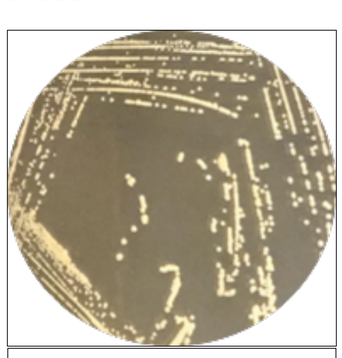


***Figure 1: A. Baumannii* ATCC 19568 colony on MHA plate**

2. Methods

A total of 45 clinical isolates (T1-T45 strains) collected from wounds and the respiratory tract of infected patients were provided by Public Health Wales (PHW). The following tests were performed;

A. Phenotypic characterization : Gram stain, Oxidase test, Catalase test, MacConkey agar, Haemolysis test, Citrate test, Motility test, Incubation at 44^0C and in CO_2

B. Genotypic characterization:

- Polymerase Chain Reaction (PCR) $Bla_{OXA\text{-}51}$ gene primers- species specific ID
- $Bla_{OXA\ 23}$ (marker of antibiotic resistance) and Class 1 integrase (*Int 1)* genes.

C. Antibiotics susceptibility: Disk diffusion assay- using EUCAST standard to test for Imipenem, Meropenem, Ciprofloxacin, Levofloxacin, Amikacin, Gentamicin, Netilmicin, Tobramycin.

D. Honey susceptibility: Well diffusion assay

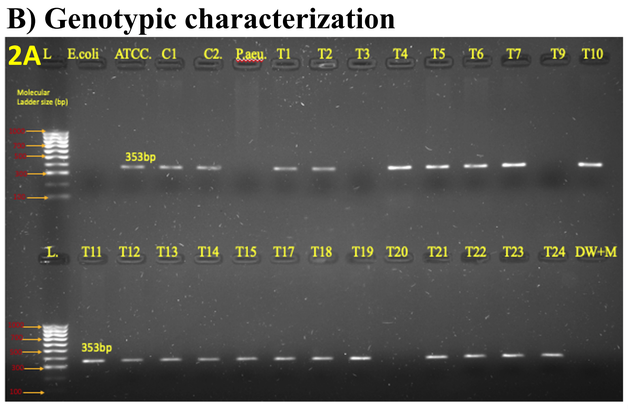
3. Results

A) Phenotypic characterization

Table 1 below shows typical results for *A. baumannii.* Using these phenotypic tests we provisionally identified 40 isolates as *A. baumannii.*

Phenotypic test	Gram Stain	Fermentation Test	Citrate test	Haemolysis test	Motility test	Catalase test	Oxidase test	Incubation at CO2	Incubation at 44 ^{0}C
Phenotypic Characteristics	Gram-negative coccobacilli	Non-Lactose fermenter	Positive	Non-haemolysis (Gamma Haemolysis)	Non motile	Positive	Negative	Strict aerobe	Grow at 37 ^{0}C and 44 ^{0}C

B) Genotypic characterization



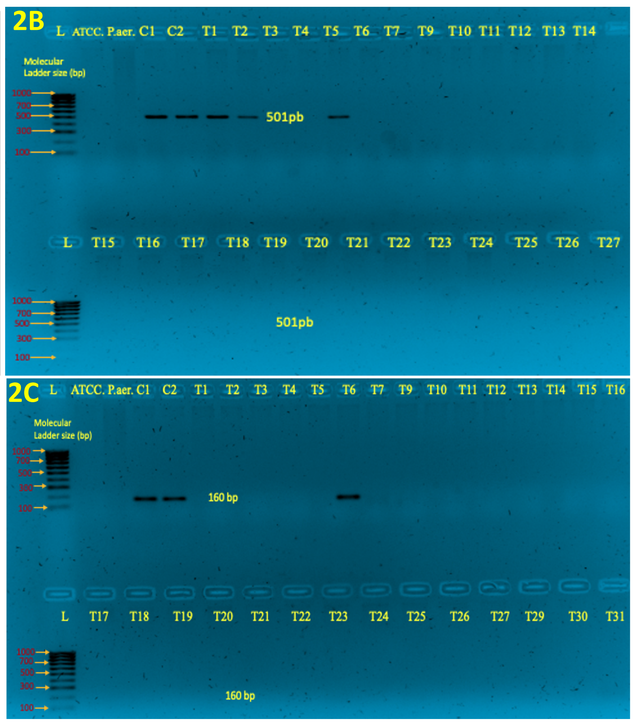


Figure (2A) $Bla_{OXA\text{-}51}$ DNA primers specific for *A. baumannii* were used to screen the isolates to confirm their identity. (2B) $Bla_{OXA\text{-}23}$ DNA primers were used for further differentiate between the various strains of *A. baumannii*. (2C) Class 1 integrase primers used for further differentiation of the clinical isolates of *A.baumannii*

Subdividing *A. baumannii* isolates on the basis of PCR results. Group 1 contains all the three genes ($bla_{OXA\text{-}51}$, $bla_{OXA\text{-}23}$ and *Int1*) (2.9%) whereas group 2 tested positive for only ($bla_{OXA\text{-}51}$ and $bla_{OXA\text{-}23}$) (31.4%), group 3 is positive only for ($bla_{OXA\text{-}51}$ and *Int1*) (2.9%) and group 4 contains only *the* $bla_{OXA\text{-}51}$ gene (62.8%).

Table 2: The four groups of *A. baumannii* classified according to their genotypes

Group	Bla_{OXA51}	Bla_{OXA23}	Int1	Number of isolates 43(100%)	Isolates
1	+	+	+	1 (2.9%)	T41
2	+	+	-	11 (31.4%)	-
3	+	-	+	1 (2.9%)	T6
4	+	-	-	22 (62.8%)	-

C) Antibiotics susceptibility

Thirty-five of the 40 isolates identified on the basis of phenotype were confirmed as *A.baumannii* based on genotypic testing. Table 3 summarises the antibiotic profiles of the different genotypes. Nine of the 11 isolates with the $bla_{OXA\ 23}$ marker were multidrug resistant (MDR) while the remaining two isolates were resistant to three of the antibiotics tested. The two *A.baumannii* isolates that contained the *Int1* gene were found to be MDR.

Table 3: Representative isolates of *A. baumannii* selected on the basis of their, genetic and antibiotic sensitivity differences for further testing with honey samples.

Samples	PCR results			Antibiotic sensitivity	Antibiotic classes
	blaOXA-51	blaOXA-23	Int1		
T11	+	-	-	R to NET & TOB only	Aminoglycosides
T40	+	+	-	R to NET	Aminoglycosides
T4	+	-	-	R to all	Carbapenems, Fluoroquinolones, Aminoglycosides
T6	+	-	+	R to all	
T41	+	+	+	R to all	

Strains with different antibiotic profiles as well as various genotypic markers were selected for further testing against our in-house collection of honey samples.

D) Honey susceptibility (Well diffusion assay)

The sensitivity of the three control strains including *A. baumannii* ATCC 19568, OXA-23 clone1 and OXA-23 clone2 were first tested against 270 honey samples to identify samples worthy of further study.

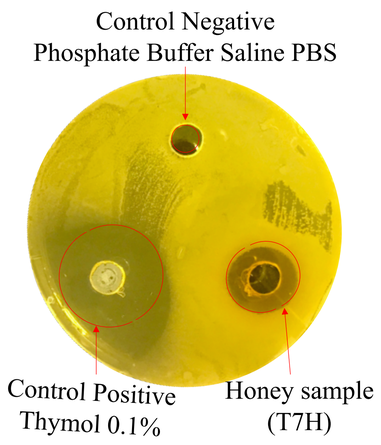


Figure 3: Well diffusion assay

Table 4: Number of honey samples in each range of diameter (mm) of zone of inhibition normalised against thymol control (0.1%) in each culture plate

Normalization diameter of zones of inhibition (mm)		Number of honey samples with activity against the test bacterium		
		(ATCC 19568)	(OXA-23-1)	(OXA-23-2)
	0.0 – 1.9	70	49	24
	2.0 – 3.9	95	82	70
Least Active	4.0 – 5.9	54	106	123
	6.0 – 7.9	36	35	54
	8.0 – 9.9	20	3	5
	10.0-11.9	2	1	0
Most Active	12.0 -12.9	0	1	1

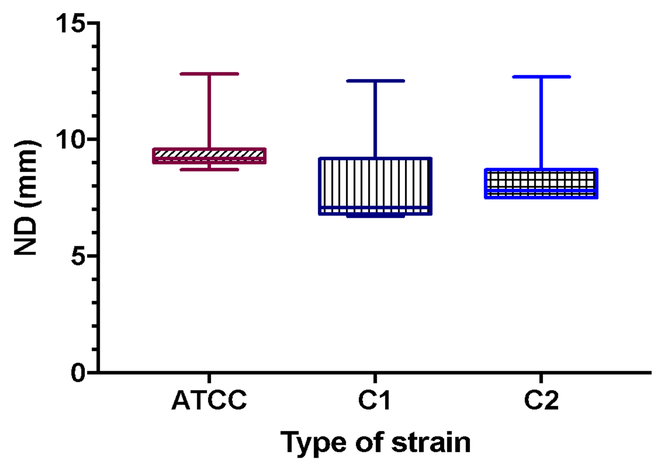


Figure 4 shows that ATCC 19568 was more sensitive (P<0.001) to honey than the MDR strains *A. baumannii* OXA 23 Clone 1 and clone 2.

The 15 most active honey samples from the in-house collection have been selected for further testing against the 6 isolates of *A. baumannii* shown in Table 3.

4. Discussion and Conclusion

The controls used in this study *A. baumannii* ATCC 19568, OXA -23 C1 and C2 confirmed the presence of all three genetic markers including $Bla_{OXA\ 51}$, $Bla_{OXA\ 23}$ and *Int*1. Using a combination of phenotypic and genotypic markers only 35 of the 40 isolates provided by PHWs were identified as *A. baumannii.* These isolates were subdivided into 4 distinct genetic groups. Nine of the 11 (82%) isolates that had both $Bla_{OXA\ 51}$, and $Bla_{OXA\ 23}$ genes were resistant to all tested antibiotics.

Upon preliminary testing of the in-house honey sample collection the *A. baumannii* OXA -23 C1 and C2 isolates were found to be significantly more resistant to honey than ATCC 19568. Using these isolates the 15 most antibacterial honey samples were identified and will be used to determine the honey sensitivity of the five clinical isolates of *A. baumannii* identified based on their genetic and antibiotics sensitivity profiles.

5. Future work

Honey susceptibility testing

1. Determine inhibitory activity of honey samples against our test panel of clinical isolates
2. Determine if any of the honey samples act synergistically with antibiotics
2. Identify novel antibacterial compounds in honey samples identified during this study

6. References

1. de Breij, A., Dijkshoorn, L., Lagendijk, E., van der Meer, J., Koster, A., Bloemberg, G., Wolterbeek, R., van den Broek, P. and Nibbering, P., 2010. Do biofilm formation and interactions with human cells explain the clinical success of Acinetobacter baumannii?. PloS one, 5(5), p.e10732.
2. Ainsworth, S. et al. 2017. Vaccination with a live attenuated Acinetobacter baumannii deficient in thioredoxin provides protection against systemic Acinetobacter infection. *Vaccine* 35(26), pp. 3387–3394. doi: 10.1016/j.vaccine.2017.05.017.
3. Antunes, L.C.S. et al. 2014. Acinetobacter baumannii: Evolution of a global pathogen. *Pathogens and Disease* 71(3), pp. 292–301. doi: 10.1111/2049-632X.12125.