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ORIGINAL ARTICLE

Detection of Quorum Sensing *N*-Acyl-Homoserine Lactone Molecules Produced by Different Resistant *Klebsiella pneumoniae* Isolates Recovered from Poultry and Different Environmental Niches

Reham A. Hosny[1] · Mai A. Fadel[2]

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Abstract

This study aimed to detect and identify the *N*-acyl-homoserine lactones molecules (AHLs) produced by different resistant *Klebsiella pneumoniae* isolates recovered from poultry and environmental samples using a modified validated high-performance liquid chromatography method. A total of 56 *K. pneumoniae* isolates were recovered, investigated for their antibiotic susceptibility, and screened for AHLs production using the *Agrobacterium tumefaciens* NTL4 biosensor system and a validated high-performance liquid chromatography method. The results revealed the detection of different short- and long-chain AHLs molecules among 39 *K. pneumoniae* isolates recovered from poultry and environmental samples. All environmental isolates produced nine peaks with retention times for C4-HSL, C6-HSL, C12-HSL, C8-HSL, C14-HSL, C8-oxo-HSL, C10-HSL, C6-oxo-HSL, and C7-HSL. The most quantifiable AHL signal molecules in poultry isolates were C4-HSL, C6-HSL, and C12-HSL. No statistical correlation between the AHL-producing ability of *K. pneumoniae* isolates and antibiotic resistance was reported. To the best of our knowledge, this study provides the first detailed report on the detection and identification of AHLs in *K. pneumoniae* isolates recovered from poultry and environmental samples. Furthermore, it provides a new insight available tool other than LC-MS/MS for detection and identification of AHL molecules.

Keywords Quorum · HPLC · Lactones · *Klebsiella pneumoniae* · Antibiotic

✉ Reham A. Hosny
rehamhosny87@yahoo.com

[1] Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Agriculture Research Center, Giza, Egypt

[2] Pharmacology and Pyrogen Unit, Department of Chemistry, Toxicology and Feed Deficiency, Animal Health Research Institute, Agriculture Research Center, Giza, Egypt

Introduction

Klebsiella pneumoniae is one of the opportunistic Enterobacteriaceae that hampers the realization of the full potential of poultry production, resulting in significant production losses or even deaths [1]. This pathogen can cause significant numbers of serious community-acquired infections, including pneumonia, urinary system infections, septicemia, and pyogenic liver abscess [2]. *K. pneumoniae* can thrive in a range of environmental niches through fecal shedding, including surface water, soil, and plant matter sewage; thus, it can pose a risk for the colonization of livestock and human [3].

Antibiotic-resistant strains of *K. pneumoniae* causing infections in humans and other animal species are becoming increasingly prevalent infections; this is related to the presence of some adherence factors that allow bacteria to persist in the environment within biofilm communities and provide protection for bacterial cells from different antimicrobials. These communities at which bacteria can be in close contact with each other presumably increased the need to use a chemical signaling communication to coordinate their activities [4, 5].

K. pneumoniae often colonize the mucosal surfaces of the nasopharynx and gastrointestinal tract. Once the host immune system is weak, it gains entry into other tissues causing infection. The colonization of bacteria to host cells is a collaborative effort task via the quorum sensing (QS) mechanism; it is often impossible for a single cell to achieve this goal [6]. QS is a complex cell-cell communication system employed by bacteria to control the expression of genes involved in certain physiological processes such as motility, virulence, biofilm formation, and drug resistance. The QS mechanism depends on the production of extracellular signaling molecules called autoinducers at a population density-dependent manner; among which *N*-acyl-homoserine lactones (AHLs) are the most commonly produced by gram-negative bacteria [7]. These molecules are characterized by the presence of a conserved lactone moiety and a variable fatty acyl side chain (between 4 and 18 carbons) [8]. The acyl side chain may be substituted with either hydroxy or oxo group at the C-3. AHLs are usually produced by LuxI enzymes (AHL synthases), and once the concentration of the AHL molecules reaches the threshold level, they bind to a LuxR response regulator, forming a complex that regulates the expression of genes involved in survival functions [7]. Many biological functions controlled by QS are associated with the pathogenicity and virulence of *K. pneumoniae* such as the production of extracellular capsular polysaccharides, biofilm formation, iron binding, and bactericidal activities [6].

Previous studies on *K. pneumoniae* isolated from different samples of the human body, such as the posterior dorsal surface of the tongue, sacral wound, burn wound, and urological specimens, have revealed the production of different types of AHLs such as C4-HSL, C6-HSL, C8-HSL, C10-HSL, and C12-HSL [7–10]. To date, there have been no published reports of AHLs production by *K. pneumoniae* recovered from poultry and environmental samples.

The most common methods used for detection and identification of AHLs involve a combination of biosensors such as *Chromobacterium violaceum* CV026, *Agrobacterium tumefaciens* NTL4(pZLR4), *Escherichia coli* pSB401, and *Escherichia coli* MG4 (pKDT17); thin-layer chromatography (TLC); and liquid chromatography–mass spectrometry (LC-MS/MS) [8, 11, 12]. The LC-MS/MS method is a sophisticated technique requiring well-trained workers and equipment that is not accessible in most developing countries. On this basis, this study aimed to detect and identify *N*-acyl-homoserine lactones molecules produced by different resistant *K. pneumoniae* strains isolated from poultry and environmental samples using a simple validated HPLC method as a basis for use in the laboratory detection of AHL signals.

Material and Methods

Samples

A total of 1465 pooled samples from eggs, organs, cloacal swabs, and different environmental niches were outlined as follows:

1. Around 390 egg samples of different origin (230 chickens, 60 ducks, 50 geese, 50 pigeons) were collected from 78 different markets in the Giza governorate and classified into 275 table eggs and 115 eggs containing dead in-shell embryos.
2. Around 480 organ samples (trachea, lung, heart, and liver) of different origins (310 chickens, 70 ducks, 50 turkeys, 50 pigeons) were collected from 96 different farms with a history of enteritis in the Giza governorate.
3. Around 345 cloacal swab samples of different origins (ducks and pigeons) were collected from 69 different backyards with a history of enteritis in the Giza governorate.
4. Around 250 environmental samples including 200 litter samples (50 chickens, 100 ducks, and 50 turkeys) and 50 water samples collected from 50 different farms in the Giza governorate.

Samples were aseptically collected immediately after processing and transported to the Reference Laboratory for Veterinary Quality Control on Poultry Production, Dokki (RLQP), for isolation of *K. pneumoniae*.

Isolation and Identification of *K. pneumoniae*

The isolation of *K. pneumoniae* was done as described by Hamza et al. [13] through the enrichment of each sample in a sterile bag containing buffered peptone water (Oxoid Limited, Thermo Fisher Scientific Inc., UK) and incubated at 37°C for 24 h. After that, a loopful of enriched samples was streaked onto MacConkey agar plates (Oxoid Limited, Thermo Fisher Scientific Inc., UK) and incubated at 37°C for 24 h. The plates were examined for the presence of mucoid and pink colonies. The identification of *K. pneumoniae* was done as described by Hansen et al. [14] using the API20E system (BioMerieux, Marcy l'Etoile, France). Isolates were preserved at −20°C in buffer peptone water containing 20% glycerol.

Antimicrobial Susceptibility

To determine the antibiotic resistance of 56 *K. pneumoniae* isolates, each isolate was inoculated onto Muller–Hinton agar (Oxoid Limited, Thermo Fisher Scientific Inc., UK) and incubated at 37°C for 24 h. The disc diffusion technique was conducted according to Clinical and Laboratory Standard Institute guidelines [15] using ten commercial discs (Oxoid Limited, Thermo Fisher Scientific Inc., UK). The antimicrobial agents and corresponding concentrations used in the study included cefotaxime (30 μg), cefaclor (30 μg), cefoxitin (30 μg), amoxicillin-clavulanic (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), tetracycline (30 μg), gentamycin (10 μg), imipenem (10 μg), and ciprofloxacin (5 μg).

Screening of AHL-Producing *K. pneumoniae* by *A. tumefaciens* NTL4 Biosensor

The AHL-producing ability of the 56 *K. pneumoniae* isolates was assayed as described by Yin et al. [7]. Each isolate was streaked on lauryl broth (LB) agar plates (Oxoid Limited, Thermo Fisher Scientific Inc., UK) containing 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (60 μg/ml) (Sigma-Aldrich, Egypt) seeded with an overnight-grown *A. tumefaciens* NTL4(pZLR4). *A. tumefaciens* NTL4(pZLR4) was previously cultured in LB agar plates supplemented with 150 μg/ml of gentamicin (Sigma-Aldrich, Egypt) and 0.5%, w/v, of glucose (Sigma-Aldrich, Egypt) to maintain the plasmid. The reporter strain was used as a negative control, and the reporter strain in a plate containing 25 nM *N*-butyryl-DL-homoserine lactone standard (C4-HSL) (Santa Cruz Biotechnology, Inc., Canada) was used as a positive control. The plates were then incubated for 48 h at 28°C. AHLs were detected by blue coloration on the plates.

Reverse-Phase High-Performance Liquid Chromatography (HPLC)

Chemicals and Reagents

Acetonitrile (ACN), methanol (MeOH) of HPLC grade, and ethylenediamine tetraacetic acid (EDTA) were purchased from Merck (Darmstadt, Germany). Analytical grade glacial acetic acid and ethyl acetate were obtained from Fischer Scientific (Leicestershire, UK). Deionized water was obtained using an ultra-pure water purification system (Millipore Sigma, Burlington, MA, USA).

Standards

Nine standards *N*-acyl-homoserine lactone compounds were obtained from Santa Cruz Biotechnology, Inc., Canada. The AHLs used were *N*-butyryl-DL-homoserine lactone (C4-HSL), *N*-hexanoyl-DL-homoserine lactone (C6-HSL), *N*-(3-oxo)-hexanoyl-DL-homoserine lactone (3-oxo-C6-HSL), *N*-heptanoyl-DL-homoserine lactone (C7-HSL), *N*-octanoyl-DL-homoserine lactone (C8-HSL), *N*-(3-oxo)-octanoyl-L-homoserine lactone (3-oxo-C8-HSL), *N*-decanoyl-DL-homoserine lactone (C10-HSL), *N*-dodecanoyl-DL-homoserine lactone (C12-HSL), and *N*-tetradecanoyl-DL-homoserine lactone (C14-HSL).

HPLC Instrumentation

Chromatography was performed using an Agilent Series 1200 HPLC system (Agilent Co., Santa Clara, CA, USA) equipped with a quaternary gradient pump, autosampler, and UV visual detector. The data acquisition was performed using an HPLC 2D ChemStation software (Hewlett-Packard, Les Ulis, France). Chromatographic separation was done using the Agilent C18 column (4.6-mm internal diameter, 150 mm, 5-μm particle size).

Preparation of Standard Solutions of AHLs

Each commercially available nine AHLs standards were dissolved in acetonitrile to obtain 1-mg/ml solutions. To obtain stock mixture solutions of 0.5-mg/ml, 50 μL of each AHLs standards (1 mg/ml) was added to 450 μL of methanol–water (35:65, v/v). After that, the

AHLs stock mixtures were further diluted in 35% methanol to obtain a series of working solutions with concentrations of 5, 10, 20, 30, 40, and 50 μg/ml for calibration. A specific volume of C4-HSL standard solution (100 μl) was added to each of these standard mixture solutions to achieve a final internal standard concentration of 10 ng/ml.

Preparation of AHL Extracts

The extraction of AHLs was performed as described by Lade et al. [16] with some modifications. A total of 56 *K. pneumoniae* isolates were grown overnight in 15 ml of LB medium buffered with 50 mM 3-(*N*-morpholino) propane sulfonic acid (MOPS) (pH 5.5) (Santa Cruz Biotechnology, Inc., Canada). The isolates were then ultracentrifuged at 10,000 g for 5 min, and the supernatant was extracted twice with 15 ml of acidified ethyl acetate (0.1%, v/v, glacial acetic acid). After that, the mixture was mixed on a shaker at 200 rpm for 2 h at ambient temperature and centrifuged at 112 g for 3 min. The upper organic layer was then separated and dried by a nitrogen evaporator at 40°C. The residues were dissolved in 250 μL of ACN and 1 mM EDTA/MeOH (1:1, v/v) and used for chromatographic analysis.

Chromatographic Conditions

Chromatography was performed with an Agilent Series 1200 HPLC system with column temperature at 35°C, flow rate 1 ml/min, and injection volume 10 μl. The chromatograms were detected on visible ultraviolet (VIS-UV) detector at 210 nm. The elution conditions were adjusted as an isocratic mobile phase of methanol:water (35:65, v/v) for 5 min, followed by a gradient mode of methanol:water (80:20, v/v) for 10 min, and followed by a linear gradient mode of 65% methanol in water for 15 min. A subsequent isocratic mode of methanol:water (35:65, v/v) for 8 min was used for flushing the column for the following run.

Validation

The developed method was validated according to ICH Q2 (R1) and USP guidelines [17, 18] for certain parameters such as linearity, sensitivity, specificity, precision, accuracy, robustness, and system suitability test.

Linearity The linearity of the method was determined by six injections of nine AHL standard mixtures (5–50 μg/ml) spiked with a working solution of internal standard (C4-HSL) (10 ng/ml). Calibration plots were obtained by plotting the normalized peak areas for each concentration. The linearity was determined using the least-squares regression equation by calculating the slope, y intercepts, and correlation coefficient (R^2).

Sensitivity It was measured in terms of detection limit (DL) and quantification limit (QL) for AHL standards. DL and QL were calculated based on the standard deviation of the response and slope of the calibration curve. DL and QL were expressed as $3.3 \times \sigma/S$ and $10 \times \sigma/S$, respectively, where "σ" is a standard deviation of y intercepts of regression lines and "S" is a slope of the calibration curve.

Specificity It was determined by comparing specific retention times of extracted samples with AHL standards chromatograms.

Precision It was evaluated by intraday and interday repeatability experiments. Intraday precision was carried out by spiking three different concentrations (10, 20, and 30 μg/ml) of AHL standards at three replicates on the same day. The interday precision was performed on three different days, assaying three replicates each day. The peak areas in the intraday and interday precisions were expressed as a percent relative standard deviation (RSD%).

Accuracy The accuracy of the developed method was evaluated by spike recovery studies using the standard addition method. It was performed through spiking three known concentrations of AHL standards (10, 20, and 30 μg/ml) in three replicates at three levels (50%, 100%, and 150%) with a quality control sample that expressed four types of AHLs which were C4-HSL, C6-HSL, C12-HSL, and C14-HSL in concentrations of 4.87, 5.92, 4.6, and 0.7 μg/ml. The calculation for recovery was performed by comparing the initial concentration of the quality control sample and actual concentrations of the spiked sample. The accuracy must not exceed 1%.

Robustness It was determined by three injections of 10 μg/ml of AHL standards under deliberate variations in method conditions such as the volume of mobile phase, detection wavelength, flow rate, and column temperature to evaluate the reliability of the method during analysis time. The volumes of methanol:water in the mobile phase were varied in the range of ±0.2% in all time points. Furthermore, the flow rate was changed (±0.005 ml/min). The detection wavelength and column temperature were also changed (±3 nm). The peak areas in the robustness were expressed as a percent relative standard deviation (RSD%).

System Suitability Test (SST) It was performed by three injections of 10 μg/ml of AHL standards to evaluate the suitability and effectiveness of the entire chromatographic system not only before use but also during analysis time. The most important investigated SST parameters were repeatability (RSD: relative standard deviations of peak response and retention time), symmetry factor, tailing factor, and theoretical plates.

Statistical Analysis

The calculations of means, standard deviations, and RSD% in the validation method were performed using Excel software (Microsoft Inc., USA). All statistical analyses were performed using IBM SPSS Statistics version 21.0 (Armonk, NY, IBM Corp.). Odds ratio analysis was used for studying the correlation between the individual antimicrobial resistance and source level. Spearman correlation analysis was performed for studying the relationship between the AHL-producing ability of *K. pneumoniae* and antibiotic resistance.

Results

Isolation and Identification of *K. pneumoniae*

K. pneumoniae was recovered in 280 samples out of 1465 from the diverse samples (eggs, organs, swabs, environment) in a percentage of 5.8%, 4.8%, 3.4%, and 5.1%, respectively,

representing 56 isolates (Table S1). Out of 390 examined egg samples, *K. pneumoniae* was isolated from the shell, albumin, yolk, and dead in shell embryo in a percentage of 6.4%, 2.6%, 9%, and 3.8%, respectively. It was found that mostly isolated *K. pneumoniae* from eggs were from the table eggs (18%) (70/390). The present study showed that *K. pneumoniae* was also mainly recovered from chicken eggs in a percentage of 10.3% (40/390) (Table S2). The API20E analysis of the putative suspected *Klebsiella* species colonies revealed that they were resolved to be *K. pneumoniae* (Table S3).

Antimicrobial Susceptibility

Frequent resistance to the ten antimicrobials tested was displayed in all 56 isolates of *K. pneumoniae* (Tables S4). In particular, *K. pneumoniae* isolates were mainly resistant to cefotaxime and tetracycline in a percentage of 93% and 50%, respectively, and mainly susceptible to ciprofloxacin and imipenem in a percentage of 94.6% and 89.3%, respectively.

K. pneumoniae antimicrobial susceptibility results were stratified by isolate source. *K. pneumoniae* isolates recovered from eggs and swabs were highly resistant to cefotaxime in a percentage of 94.1% and 90%, respectively, and tetracycline in a percentage of 58.8% and 50%. *K. pneumoniae* isolates recovered from organs and environment were highly resistant to cefotaxime in a percentage of 85.7% and 100%, respectively, and nalidixic acid in a percentage of 64.3% and 40%, respectively. A statistically significant correlation between the resistance of chloramphenicol antimicrobial and isolate source level was reported; *K. pneumoniae* isolates recovered from poultry organs were 6.5 times more likely to develop chloramphenicol resistance than those isolated from environmental source (odds ratio = 6.5, 95% confidence interval = 1.053–40.132, P value = 0.033).

Screening of AHL-Producing *K. pneumoniae* by *A. tumefaciens* NTL4 Biosensor

A total of 39 *K. pneumoniae* isolates (26 poultry and 13 environmental) were found to produce AHLs based on the development of blue coloration resulting from hydrolysis of X-gal in the presence of *A. tumefaciens* NTL4(pZLR4) reporter strain.

HPLC Method Validation

The developed method was validated through different parameters that were found to be within the acceptance limit.

Linearity

The calibration curves for AHL standards displayed a linear relationship over the range of concentrations from 5 to 50 μl/ml in six replicates. The correlation coefficients (R^2) for the nine AHLs standards ranged from 0.9996 to 1 (Table 1).

Sensitivity

LOD and LOQ for AHLs were found to range from 0.16–1.8 μg/ml and 0.48–5.45 μg/ml, respectively (Table 1).

Table 1 Specificity, linearity, intraday and interday precision, recovery, accuracy, and sensitivity

AHLs analysis	Specificity (min)	Regression equation & correlation coefficient (R^2)	Precision (RSD%) (n = 3)		Recovery %	Accuracy% (mean ± SD)	Sensitivity	
			Intraday	Interday			LOD (μg/ml)	LOQ (μg/ml)
C4-HSL	3.459	Y = 1.4012x + 0.2656 R^2 = 0.9999	0.6–0.77	0.77–0.92	100.48–101.46%	101.04 ± 0.47	0.96	2.9
C6-HSL	5.913	Y = 1.0022x + 0.107 R^2 = 0.9998	0.475–0.55	0.3–0.81	99.47–101.96%	100.9 ± 0.98	1.8	5.45
C7-HSL	8.351	Y = 1.2132x + 0.135 R^2 = 1	0.48–0.87	0.42–0.79	99.51–100.93	100.404 ± 0.52	0.92	1.27
3-oxo-C6-HSL	10.174	Y = 2.1837x + 0.0174 R^2 = 0.9998	0.23–0.73	0.41–0.48	98.38–100.17	99.22 ± 0.7	1.5	4.64
3-oxo-C8-HSL	10.931	Y = 2.8149x + 0.0636 R^2 = 0.9996	0.35–0.74	0.36– 1	98.49–100.4	99.1 ± 0.77	1.42	4.3
C8-HSL	14.463	Y = 2.068x + 0.0943 R^2 = 0.9998	0.42–0.79	0.73–1.01	99.85–101.79	101.03 ± 0.7	1.7	5.1
C10-HSL	20.531	Y = 3.3759x + 0.9014 R^2 = 0.9998	0.32–0.62	0.41–0.86	100.71–102	101.75 ± 0.87	1.6	4.9
C12-HSL	24.941	Y = 1.6091x + 0.0714 R^2 = 0.9999	0.57–0.64	0.5–0.99	99.64–100.85	100.4 ± 0.5	1.3	3.9
C14-HSL	29.532	Y = 1.5011x + 0.004 R^2 = 1	0.33–0.68	0.54–0.79	99.95–100.2	100.02 ± 0.8	0.16	0.48

AHLs, *N*-acyl homoserine lactones; *RSD*, relative standard deviation; *SD,* standard deviation; *LOD*, limit of detection; *LOQ*, limit of quantification

Specificity

There were no significant interfering peaks observed from the retention times corresponding to AHL standards and extracted samples. The resulting retention times for AHLs were expressed in minutes (Table 1 and Fig. 1a, b).

Precision

Intraday and interday precision results were calculated from a residual standard deviation of the regression line and expressed by RSD%. The RSD% values of intraday and interday tests of this developed method ranged from 0.23 to 0.87% and 0.3 to 1.01%, respectively (Table 1).

Accuracy

The accuracy and recovery of spiked samples with AHL standards were tested at 10, 20, and 30 μg/ml. The accuracy of AHLs ranged from 99.1% ± 0.77 to 101.75% ± 0.87 for all tested

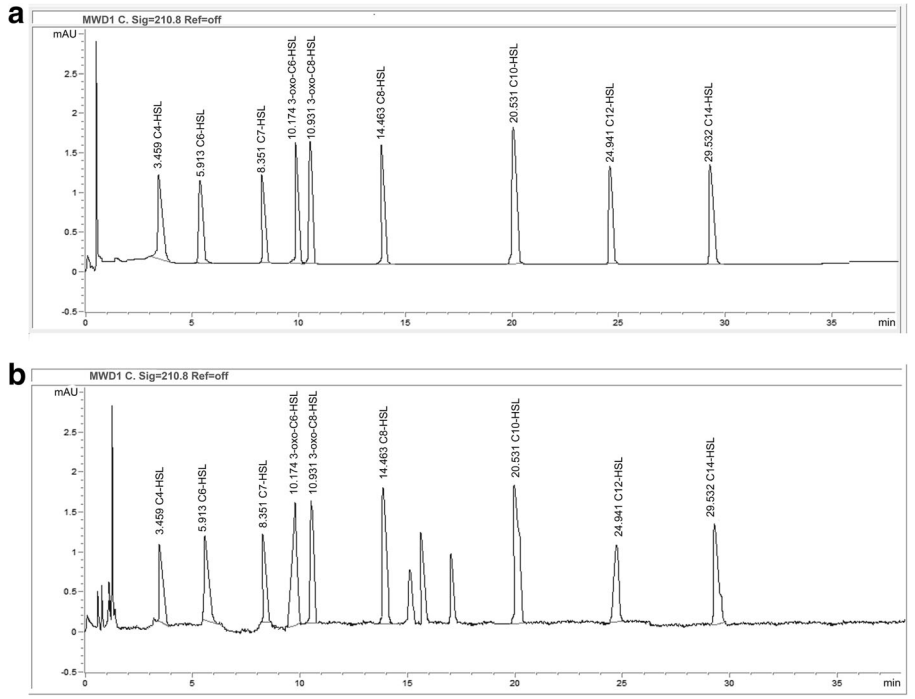

Fig. 1 **a** HPLC chromatogram of nine AHL standards at a concentration of 5 μg/ml. AHL standards expressed nine peaks with retention times of 3.459 min for C4-HSL, 5.913 min for C6-HSL, 10.174 min for 3-oxo-C6-HSL, 14.463 min for C8-HSL, 10.931 min for 3-oxo-C6-HSL, 20.531 min for C10-HSL, 24.941 min for C12-HSL, and 29.532 min for C14-HSL. **b** HPLC chromatogram of AHLs extracted from a water sample with an average concentration of 5.18 μg/ml. The water sample expressed nine peaks with retention times of 3.459 min for C4-HSL, 5.913 min for C6-HSL, 10.174 min for 3-oxo-C6-HSL, 14.463 min for C8-HSL, 10.931 min for 3-oxo-C6-HSL, 20.531 min for C10-HSL, 24.941 min for C12-HSL, and 29.532 min for C14-HSL are identical to AHL standards

concentrations, while the recovery of AHLs ranged from 97.03 to 103.01%, 98.3 to 105.03%, and 96.3 to 102.04% regarding each concentration, respectively (Fig. 2a, b) (Table 1).

Robustness

The RSD% values of peak areas obtained from injections of AHL standards at different method conditions (methanol volume of the mobile phase, detection wavelength, flow rate, and column temperature) ranged from 0.92 to 2.02% (Table 2).

System Suitability Test

The results obtained by the developed method have fulfilled the parameters of system suitability. The RSD% values for retention times and peak areas for the AHL standards ranged from 0.02 to 0.6% and 0.05 to 0.8%, respectively. The peak symmetries of AHL standards were indicated by RSD% values of tailing and symmetry factors that ranged from 0.01 to 0.7% and 0.01 to 0.8%, respectively. Mean values for the theoretical plates of AHL standards ranged from 9057.4 to 9983.4 (Table 2).

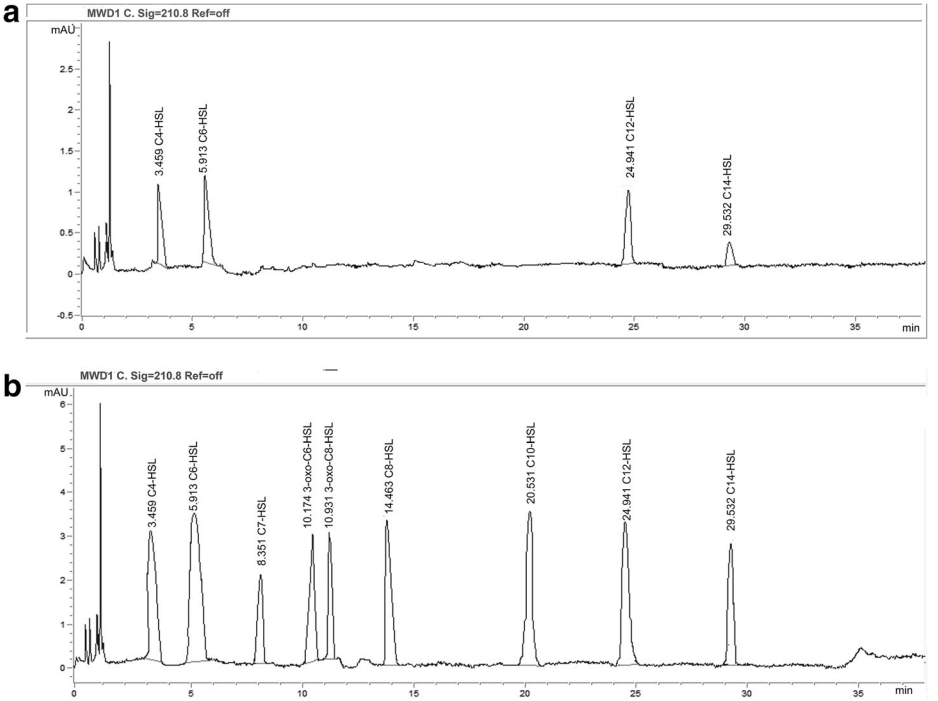

Fig. 2 **a** HPLC chromatogram of quality control sample before spiking with 10 μg/ml of AHLs standards. The quality control sample expressed four types of AHLs which were (C4-HSL, C6-HSL, C12-HSL, and C14-HSL) in concentrations of 4.87, 5.92, 4.6, and 0.7 μg/ml, respectively. **b**. HPLC chromatogram of quality control sample spiked with 10 μg/ml of AHL standards. A quality control sample that expressed four types of AHLs (C4-HSL, C6-HSL, C12-HSL, and C14-HSL) was spiked with 10 μg/ml of AHL standards. The calculation for recovery was performed by comparing the initial concentration of the quality control sample and actual concentrations of the spiked sample

Table 2 Robustness and system suitability test parameters on a spike concentration of 10 μg/ml (n = 3)

	Robustness (pooled RSD%)	System suitability test				
		Theoretical plates	Mean ± RSD		Symmetry factor	Tailing factor
			Repeatability			
			Retention time	Peak area		
C4-HSL	1.83	9173.3 ± 0.3	3.457 ± 0.4	14.01 ± 0.8	0.92 ± 0.02	1.4 ± 0.01
C6-HSL	2.02	9331.2 ± 0.16	5.915 ± 0.11	10.01 ± 0.3	0.82 ± 0.01	1.04 ± 0.1
C7-HSL	0.92	9204.3 ± 0.08	8.351 ± 0.02	12.03 ± 0.45	0.91 ± 0.3	1.13 ± 0.2
3-oxo-C6-HSL	1.58	9057.4 ± 0.04	10.171 ± 0.2	21.8 ± 0.1	0.84 ± 0.8	1.3 ± 0.7
3-oxo-C8-HSL	1.61	9178.1 ± 0.04	10.929 ± 0.4	27.8 ± 0.05	0.9 ± 0.2	1.4 ± 0.5
C8-HSL	1.93	9633.3 ± 0.3	14.463 ± 0.2	20.63 ± 0.6	0.97 ± 0.5	1.05 ± 0.1
C10-HSL	1.37	9518..2 ± 0.3	20.53 ± 0.6	33.51 ± 0.3	0.83 ± 0.2	1.07 ± 0.04
C12-HSL	1.23	9705.1 ± 0.1	24.941 ± 0.07	16.01 ± 0.3	0.91 ± 0.05	1.14 ± 0.1
C14-HSL	0.95	9983.4 ± 0.4	29.532 ± 0.05	14.9 ± 0.05	0.97 ± 0.1	1.04 ± 0.04
Acceptance criteria	> 6	>2000	RSD < 1.0%	RSD < 1.0%	< 1.0%	RSD ≤ 2.0

RSD, relative standard deviation

Detection of AHL-Producing Ability of *K. pneumoniae* Isolates Using HPLC

The HPLC chromatogram of the 56 *K. pneumoniae* isolates revealed that 39 isolates (26 poultry and 13 environmental) expressed different concentrations of AHLs at various retention times (Tables 3 and 4. All 13 environmental *K. pneumoniae* isolates expressed nine peaks, with retention times of 3.459 min for C4-HSL, 5.913 min for C6-HSL, 10.174 min for 3-oxo-C6-HSL, 14.463 min for C8-HSL, 10.931 min for 3-oxo-C6-HSL, 20.531 min for C10-HSL, 24.941 min for C12-HSL, and 29.532 min for C14-HSL, are identical to AHL standards. The most quantifiable AHL concentrations in 26 poultry *K. pneumoniae* isolates were C4-HSL, C6-HSL, C12-HSL, C8-HSL, and C14-HSL in percentages of 100% ,84.6% ,76.9%, 50%, and 50%, respectively, whereas the lowest quantifiable AHL concentrations were C8-oxo-HSL, C10-HSL, C6-oxo-HSL, and C7-HSL in percentages of 23.1% ,15.4%, 11.5%, and 7.7%, respectively (Table S5).

Relationship Between Quorum Sensing and Antibiotic Resistance

Analysis of AHLs in different resistance phenotypes of *K. pneumoniae* isolates was shown in Table S5. There was no statistical correlation between the AHL-producing ability of *K. pneumoniae* isolates and antibiotic resistance using Spearman correlation (P value = 0.874).

Discussion

The detection of bacterial strains producing QS signaling molecules has a great significance towards the discovery of novel nonantibiotic-dependent therapy especially in

Table 3 Quorum sensing signals of the 56 *K. pneumoniae* isolates recovered from different sources

Number of AHLs	Quorum sensing signals	Number of *K. pneumoniae* isolates	Total number of *K. pneumoniae* isolates (%)
0		17	17 (30.4%)
2	C4, C6	3	3 (5.4%)
3	C4, C6, C12	3	4 (7.1%)
	C4, C8, C12	1	
4	C4, C6, C12, C14	6	11 (19.6%)
	C4, 3 oxo-C6, C8, C12	2	
	C4, C6, C8, C10	1	
	C4, C6, C12, C14	1	
	C4, C6, C8, C12	1	
5	C4, C6, 3OXO-C8, C8, C12	2	4 (7.1%)
	C4, C6, 3OXO C8, C12, C14	2	
6	C4, C6, C8, C10, C12, C14	1	3 (5.4%)
	C4, C6,3 OXOC6. 3OXO C8, C8, C14	1	
	C4, C7, 3OXO-C8, C8, C10, C14	1	
7	C4, C6, C7, C8, C10, C12, C14	1	1 (1.8%)
9	C4, C7, 3OXO-C6, C6, C8,3OXO-C8, C10, C12, C14	13	13 (23.2%)

AHLs, *N*-acyl homoserine lactones; the percentage of the total number of *K. pneumoniae* isolates (%) was calculated according to the total number of examined isolates (56)

treating multidrug-resistant infections [9, 19]. Critically, the emergence of *K. pneumoniae* as a source of antibiotic resistance pathogen, in the medical and veterinary fields, presents a challenging problem for clinicians and veterinarians in terms of the treatment of humans and animals. Two major QS communication systems have been described in *K. pneumoniae*, type 1 system and type 2 system [10]. Type 1 system (LuxR/I system) regulates intraspecies communication by two proteins, LuxI, which produces the AHL autoinducer, and LuxR, which is activated by the autoinducer and regulates the transcription of several genes involved in certain biological functions. Type 2 system regulates interspecies communication by LuxS gene that is involved in the regulation of biofilm formation and lipopolysaccharide synthesis [10]. Until now, there is a lack of research studies on the effect of AHL autoinducer molecules on the virulence factors of *K. pneumoniae*. Furthermore, AHLs production by *K. pneumoniae* in the veterinary sector was of little attention. Hence, the present study was aimed to detect and identify AHLs molecules produced by different resistant *K. pneumoniae* isolates recovered from the poultry and environmental samples using a simple developed and validated HPLC method. The prevalence and distribution of various *K. pneumoniae* and their phenotypic resistance from different ecological habitats vary considerably from one geographic area to another, but the current direction towards globalization means that their consequences spread across geographical borders. Despite the broad history of *K. pneumoniae infections in humans*, studies on infections by this pathogen in poultry are scarce. Epidemiological investigations have reported that eggs are considered as one of the sources of foodborne infection outbreaks in humans [20]. Until now, *K. pneumoniae* is not included in the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) [21] and thus there are no current surveillance systems that monitor for this pathogen in the food supply. The present study displayed the higher

Table 4 Quantitation of AHLs in *K. pneumoniae* isolates using a validated HPLC method

Sample extract		AHLs concentrations (μg/g)								
		C4-HSL	C6-HSL	C7-HSL	3-oxo-C6-HSL	3-oxo-C8-HSL	C8-HSL	C10-HSL	C12-HSL	C14-HSL
Chicken eggs	KP1	2.1	3.4	ND	ND	ND	ND	ND	ND	ND
	KP2	7.55	7.06	ND	ND	ND	9.81	6.06	7.98	ND
	KP5	5.35	6.53	5.78	ND	ND	5.42	4.71	5.64	3.65
	KP6	2.95	4.43	ND	ND	ND	ND	ND	5.0	ND
	KP8	3.20	ND	3.60	ND	6.5	3.89	5.15	ND	3.25
Duck eggs	KP9	18.40	7.52	ND	27.87	13.74	14.99	ND	ND	13.63
	KP10	12.27	10.44	ND	ND	6.19	ND	ND	15.50	ND
Geese eggs	KP13	3.92	7.52	ND	ND	ND	ND	ND	4.96	ND
Pigeon eggs	KP14	39.02	ND	ND	ND	ND	38.79	ND	49.83	ND
	KP15	20.82	ND	ND	18.62	ND	19.23	ND	27.17	ND
	KP16	23.38	21.76	ND	ND	ND	ND	ND	29.88	ND
	KP17	2.64	ND	ND	3.84	ND	2.42	ND	3.09	ND
Mean ± SD		11.8 ± 11.5	8.6 ± 5.7	4.7 ± 1.5	16.8 ± 12.1	8.8 ± 4.3	13.5 ± 12.7	5.3 ± 0.7	16.6 ± 15.97	6.8 ± 5.9
Chicken organs	KP19	4.39	4.91	ND	ND	ND	4.04	ND	4.95	ND
	KP20	4.64	5.02	ND	ND	4.8	5.9	ND	5.1	ND
	KP23	2.32	3.42	ND	ND	ND	ND	ND	5.2	ND
	KP26	12.70	13.38	ND	ND	ND	ND	ND	14.1	1.3
	KP27	7.86	8.51	ND	ND	ND	ND	ND	10.9	1.1
Duck organs	KP29	16.98	15.48	ND	ND	8.1	ND	ND	15.9	0.54
	KP30	7.16	8.23	ND	ND	ND	ND	ND	9.8	1.2
	KP31	12.14	14.70	ND	4.9	ND	ND	ND	25.2	ND
Mean ± SD		8.5 ± 4.9	9.2 ± 4.7	ND	4.9 ± 00.00	6.5 ± 2.3	4.97 ± 1.3	ND	11.4 ± 6.97	1.04 ± 0.3
Duck swabs	KP32	7.57	7.29	ND	ND	ND	ND	ND	ND	ND
	KP33	29.01	21.99	ND	ND	ND	18.61	23.93	ND	ND
	KP37	3.03	3.98	ND	ND	ND	ND	ND	3.78	3.97
	KP38	6.53	6.30	ND	ND	ND	ND	ND	ND	ND
Pigeon swabs	KP41	9.52	11.05	ND	ND	ND	ND	ND	11.50	12.05
Mean ± SD		11.13 ± 10.3	10.12 ± 7.1	ND	ND	ND	18.61	23.93	3.06 ± 5.00	3.20 ± 5.2
Duck litters	KP42	3.5	7.30	2.805	4.999	6.86	18.19	8.59	6.60	6.1
	KP43	18.55	20.33	13.11	13.97	10.54	33.02	11.7	29.6	25.9
	KP44	3.74	8.51	6.24	5.76	6.51	42.95	7.02	6.94	6.2
	KP45	5.34	9.39	7.43	8.34	8.07	48.46	14.5	12.9	12.3
	KP46	6.84	8.94	8.713	9.39	13.76	51.27	26.3	31.4	25.3
	KP48	6.21	6.40	7.814	8.79	10.81	5.87	6.39	4.88	4.5
	KP49	4.702	7.61	6.26	6.80	11.07	33.59	23.4	36.6	39.4
	KP51	3.997	9.60	2.954	7.72	8.41	8.20	5.30	3.77	3.3
	KP52	5.497	9.40	6.788	8.88	9.48	19.45	5.02	5.61	4.0
	KP53	2.91	4.52	5.79	4.998	11.29	9.60	5.42	5.01	1.1
Chicken litters	KP54	2.71	6.39	3.69	5.30	4.81	6.30	4.98	4.42	1.4
	KP55	8.75	5.44	8.63	7.43	7.95	19.69	11.8	9.95	9.3
Mean ± SD		6.1 ± 4.3	8.7 ± 4.03	6.7 ± 2.9	7.7 ± 2.5	9.1 ± 2.5	24.7 ± 16.6	10.9 ± 7.3	13.1 ± 12.1	11.6 ± 12.1
Water	KP56	4.3	5.1	4.98	5.01	4.91	6.7	6.2	4.41	5.01

AHLs, *N*-acyl homoserine lactones; *ND*, not detected; *SD*, standard deviation

prevalence of *K. pneumoniae* in table egg samples collected from markets (82.4%) compared to dead in shell eggs that displayed a prevalence of 17.6%; this may be related

to surface contamination during handling, storage, and transportation [22]. Similarly, a study by Khan et al. [23] has displayed that members of the family Enterobacteriaceae, such as *Escherichia coli*, *Salmonella* serotypes, and *Klebsiella* species, are common causes of yolk sac infections and dead in shell. Moreover, the prevalence of *K. pneumoniae* in the shell, albumin, and yolk components of eggs in this study may be related to the infection of the ovarian follicles, magnum, and shell during egg formation, respectively [22]. A study by Rehault-Godbert et al. [24] has displayed that the temperature and time of storage play a role in the diminution of antimicrobial activity in the albumin through the degradation of ovotransferrin and ovalbumin; this explains the prevalence of *K. pneumoniae* infection in the albumin of eggs in the present study. Earlier investigations have displayed the presence of *K. pneumoniae* as normal microbiota of the gastrointestinal tract of animals and as free-living *bacteria* in the environment such as water, plants, and soil [25, 26]. The isolation of *K. pneumoniae* in organs in this study was at a prevalence of 14.6% (70/480), which was consistent with those reported in previous studies that reported a prevalence range of 10–13% [27, 28]. The isolation of *K. pneumoniae* in cloacal swabs in this study was at a prevalence of 14.5% (50/345), which was higher than those reported in a previous study in Indonesia [1] that reported a prevalence of 7.8% and lower than those in a previous study in Nigeria [29] that reported a prevalence of 21.6%. Moreover, *K. pneumoniae* was isolated from the environmental samples in a percentage of 30% (75/250) that was lower than those reported in previous studies that reported prevalence rates of 51.7% and 74.9% [30, 31]. The variation of the distribution of *K. pneumoniae* in this study compared to the results of previous studies might be related to the variations in the types and sources of samples.

A total of 43 *K. pneumoniae* isolates in this study exhibited resistance to more than one tested antimicrobial in a percentage of 76.8%. This high resistance level might be related to the abuse of antibiotics in poultry farms [32]. High rates of resistance were detected against cefotaxime, tetracycline, and nalidixic acid. Similarly, previous studies have displayed high levels of resistance to beta-lactams and tetracyclines [33, 34]. The high level of nalidixic acid resistance in this study suggests the presence of significant nalidixic acid pressure in the environment as quinolones are mainly encoded chromosomally; rare studies have displayed a low level of plasmid-mediated resistance [35]. It was surprising to record (30.4%, 32.1%, 10.7%, and 16.1%) levels of resistance to cefaclor, amoxicillin-clavulanic, imipenem, and cefoxitin, respectively, in this study as they are not commonly used in poultry farms. These results may be attributed to the transmission of resistance genes either through sewage effluents or infected human carriers [13]. Moreover, the most effective antibiotics against *K. pneumoniae* isolates in this study were found to be primarily ciprofloxacin followed by imipenem. Similarly, Hayati et al. [1] has displayed 72.7% sensitivity of *K. pneumoniae* isolated from chicken cloacal swabs to ciprofloxacin. Previous studies on *K. pneumoniae* have displayed similar observations regarding the effectiveness of imipenem and ciprofloxacin in the treatment of UTI infections in humans caused by *K. pneumoniae* [36, 37].

The ability of the 56 *K. pneumoniae* isolates to produce AHL molecules was tested using a reporter strain of *A. tumefaciens* NTL4(pZLR4). It contains a recombinant plasmid pZLR4 carrying a traG:lacZ reporter fusion that is induced when its transcription activator (TraR) detects exogenous AHLs with acyl chain lengths from 4 to 12 carbons, resulting in the production of a β-galactosidase enzyme that is measured by using X-gal through blue

coloration detection [38]. The only limitation of this assay is that the monitoring system lies within the scope of one reporter strain that gave low responsiveness to unsubstituted acyl-homoserine lactones [12]. However, in this study, it has been shown that the results of AHL detection by a reporter strain complied with HPLC results; this may be attributed to the production of high concentrations of AHLs as displayed in the HPLC detection results. Our results revealed that 69.6% of the examined *K. pneumoniae* isolates were shown to produce AHLs when grown in LB medium under aerobic conditions. Similarly, previous studies have displayed the detection of AHLs in *K. pneumoniae* strains recovered from the sacral wound and human tongue surface using *A. tumefaciens* NTL4(pZLR4) and *C. violaceum* CV026 under aerobic condition [7, 9].

The extraction of AHLs in the isolated *K. pneumoniae* is the first step towards the detection and quantification of AHLs. The optimization of AHLs extraction was applied in this study through the addition of MOPS solution into LB enrichment medium to protect AHLs against lactonolysis at alkaline pH and maximize extraction efficiency. Similarly, earlier studies have reported the incorporation of MOPS solution in the extraction of AHLs causes a sharp drop in the lactonase activity [39, 40]. Also, three protein precipitating organic solvents such as acidified ethyl acetate, methanol, and acetonitrile have been used to disrupt the protein-bound AHL complex and extract the AHL. The protein precipitation method is the most common sample extraction approach that removes the majority of the proteins from the samples and requires minimal method development [41]. Furthermore, EDTA has been used to improve the power of extraction and eliminate the matrix interference effect. Previous studies have displayed the usage of EDTA in the AHLs extraction, decreasing the hydrolysis activity of *N*-acyl-homoserine lactonases, increasing the sharpness and resolution of AHLs peak area, and chelating some metals that are usually present in all biological and environmental samples such as nickel and cadmium ions and may inhibit quorum sensing activity [42–44].

Many studies have displayed the application of the LC-MS/MS method for detection and identification of AHLs produced by different bacteria such as clinical *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Aeromonas veronii* [7, 9, 11, 19]. This is the first study concerning the use of the reversed-phase HPLC method in the detection and identification of AHLs produced by *K. pneumoniae* isolates recovered from poultry and environmental samples. HPLC analysis was performed to identify AHLs by comparing appeared peaks retention time with those of respective standard AHLs. The optimization of elution in the HPLC analysis was applied in this study through the increment of methanol:water as a mobile phase for C18-HPLC column having a pH range (7–8.1) at different concentrations; this maintains the lactone ring of homoserine lactone (HSL) in the opened phase and allows more detectable power for different AHLs. This was explained by Yates et al. [45] who reported that the opening of the lactone ring of homoserine lactone and releasing of hydroxide ions are related to the hydrolysis activity of the lactonase enzyme that has optimal activity at pH 7–8.

The selection of the UV wavelength used (210 nm) in this study was related to the ability of AHLs to absorb UV light at low wavelengths near-vacuum UV (200 nm) [46]. As HPLC method employing UV detection encounters limitations including shadowing absorbance and production of nonspecific peaks by the mobile phases of acetonitrile [47, 48]. Instead, the use of methanol in this study as a mobile phase was related to its higher UV absorbance ability in the far UV region (less than 250 nm) compared to acetonitrile [49].

This developed method was validated according to ICH Q2 (R1) and USP guidelines [17, 18] to reveal the performance characteristics, including linearity and sensitivity, specificity, precision, accuracy, recovery, robustness, and system suitability. The linearity of an analytical method is defined as the ability of the method to provide results within a specific range that are proportional to the concentration of the analyte. The coefficients of correlations (R^2) for nine AHL standards were >0.999 over the concentration range of 5 to 50 μl/ml for AHLs, indicating a linear relationship between the concentration of AHLs and peak area. The limits of detection (LOD) and quantification (LOQ) are determined as the lowest concentration of the analyte in the sample that can be detected and quantified, respectively. The detection and quantification limits of this developed method were low enough to enable the determination of AHLs. Specificity is defined as the ability to assess the target analyte in the presence of interfering components. As there was no significant impurities interference in the AHLs observed at the retention times of both the extracted samples and AHL standards, this method can be considered to be specific for AHL determination. Precision is defined as the degree of agreement of a series of measurements under variation of day. The relative standard deviations of the peak areas for both intraday and interday precisions were ≤1%, suggesting good precision of this method. The accuracy is defined as the agreement of results obtained by the developed method to the true value. The results of recovery studies in this study were within the acceptable limit for recovery (80 to 120%) at all three concentrations of AHL standards, suggesting the efficiency of these extraction and chromatography procedures for AHLs analysis. The robustness of the method was determined by studying the effects caused by minor changes in method conditions. The pooled RSD% values for the three replicates of AHL standards overconcentration of 10 μg/ml at different conditions, such as methanol volume of the mobile phase, detection wavelength, flow rate, and column temperature, were >6%, indicating the robustness of the developed method as no considerable changes in the peak areas were observed. The system suitability test is determined to assess system performance either before or during chromatographic analysis to ensure the repeatability and performance of chromatography conditions. The RSD% values for retention times and peak areas for the AHL standards were <1%, suggesting the repeatability for these parameters. The performance of chromatography conditions was assessed by different parameters such as tailing factor, symmetry factor, and a number of theoretical plates. In this method, the good peak symmetries of AHLs were indicated by RSD% values < 1% for the tailing factor and <2 for the symmetry factor, and the good efficiency of the column was indicated by the mean values of <2000 for the number of theoretical plates.

HPLC analysis of the 56 *K. pneumoniae* isolates in this study has displayed that 69.6% of the isolates produced high amounts of different AHL profiles, as displayed by peaks observed at the corresponding retention time for each type. These results may reflect on the regulation of different physiological activities in *K. pneumoniae* strains such as motility, virulence, and biofilm formation. Similarly, previous studies have reported that the types and amounts of the produced AHLs are related to the isolation of AHL-producing strains from different sites of infection or the presence of different laboratory conditions [9, 50]. Previous studies on the clinical *K. pneumoniae* isolates recovered from the posterior dorsal surface of the tongue and sacral wound swabs have displayed production of low amounts of *N*-hexanoyl-homoserine lactone (C6-HSL), *N*-octanoyl homoserine lactone (C8-HSL), and *N*-3-dodecanoyl homoserine lactone (C12-HSL) [7, 9]. Although there was no statistical correlation between the AHL-producing ability of *K. pneumoniae* isolates and antibiotic resistance, it is not

comprehensive to judge that without an experimental approach. Further experimental research studies are needed to study the link between AHLs production and antibiotic resistance.

Conclusions

This study provides a first detailed report on the detection and identification of AHLs in *K. pneumoniae* isolates recovered from poultry and environmental sources. Besides, it provides a new insight available tool other than LC-MS/MS for detection and identification of AHL molecules.

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Code Availability Not applicable.

Author Contribution Reham A. Hosny designed the workflow, performed cultural isolation of *K. pneumoniae* and biosensor detection of acyl-homoserine lactone molecules, and performed all statistical analyses throughout the whole manuscript. Mai A. Fadel developed an RP-HPLC method for the detection of *N*-acyl-homoserine lactone molecules and statistically analyzed validated method results. Reham A. Hosny and Mai A. Fadel wrote, revised, and approved the manuscript.**Data availability** The authors confirm that the data supporting the findings of this study are available within the article and its supporting information.

Declarations

Conflict of Interest The authors declare no competing interests.

References

1. Hayati, M., Indrawati, A., Mayasari, N. L. P. I., Istiyaningsih, I., & Atikah, N. (2019). Molecular detection of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolates of chicken origin from East Java, Indonesia. *Veterinary World, 12*(4), 578–583.
2. Guo, Y., Zhou, H., Qin, L., Pang, Z., Qin, T., Ren, H., & Zhou, J. (2016). Frequency, antimicrobial resistance and genetic diversity of *Klebsiella pneumoniae* in food samples. *PLoS One, 11*(4), e0153561.
3. Savin, M., Bierbaum, G., Hammerl, J. A., Heinemann, C., Parcina, M., Sib, E., & Kreyenschmidt, J. (2020). ESKAPE bacteria and extended-spectrum-β-lactamase-producing *Escherichia coli* isolated from wastewater and process water from German poultry slaughterhouses. *Applied and Environmental Microbiology, 86*(8).
4. Chen, L., Wilksch, J. J., Liu, H., Zhang, X., Torres, V. V., Bi, W., ... & Zhou, T. (2020). Investigation of LuxS-mediated quorum sensing in *Klebsiella pneumoniae*. *Journal of Medical Microbiology, 69*(3), 402, 413.
5. Koraimann, G., & Wagner, M. A. (2014). Social behavior and decision making in bacterial conjugation. *Frontiers in Cellular and Infection Microbiology, 4*, 54.
6. Liu, W., Lu, H., Chu, X., Lou, T., Zhang, N., Zhang, B., & Chu, W. (2020). Tea polyphenols inhibits biofilm formation, attenuates the quorum sensing-controlled virulence and enhances resistance to *Klebsiella pneumoniae* infection in Caenorhabditis elegans model. *Microbial Pathogenesis, 147*, 104266.

7. Yin, W.-F., Purmal, K., Chin, S., Chan, X.-Y., Koh, C.-L., Sam, C.-K., & Chan, K.-G. (2012). *N*-acyl homoserine lactone production by *Klebsiella pneumoniae* isolated from human tongue surface. *Sensors, 12*(3), 3472–3483.
8. Nievas, F., Bogino, P., Sorroche, F., & Giordano, W. (2012). Detection, characterization, and biological effect of quorum-sensing signaling molecules in peanut-nodulating bradyrhizobia. *Sensors, 12*(3), 2851–2873.
9. Ngeow, Y. F., Cheng, H. J., Chen, J. W., Yin, W.-F., & Chan, K.-G. (2013). Short chain *N*-acylhomoserine lactone production by clinical multidrug resistant *Klebsiella pneumoniae* strain CSG20. *Sensors, 13*(11), 15242–15251.
10. Mukesh, K., Harjai, K., & Chhibber, S. (2017). Prevalence of AI-1 type of quorum sensing molecules in *Klebsiella pneumoniae* clinical isolates. *International Journal of Advanced Research, 5*(7), 101–107.
11. Gui, M., Liu, L., Wu, R., Hu, J., Wang, S., & Li, P. (2018). Detection of new quorum sensing *N*-acyl homoserine lactones from *Aeromonas veronii*. *Frontiers in Microbiology, 9*, 1712.
12. Steindler, L., & Venturi, V. (2007). Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiology Letters, 266*(1), 1–9.
13. Hamza, E., Dorgham, S. M., & Hamza, D. A. (2016). Carbapenemase-producing *Klebsiella pneumoniae* in broiler poultry farming in Egypt. *Journal of Global Antimicrobial Resistance, 7*, 8–10.
14. Hansen, D. S., Aucken, H. M., Abiola, T., & Podschun, R. (2004). Recommended test panel for differentiation of *Klebsiella* species on the basis of a trilateral interlaboratory evaluation of 18 biochemical tests. *Journal of Clinical Microbiology, 42*(8), 3665–3669.
15. CLSI. (2017). Performance standards for antimicrobial susceptibility testing: Twenty-Seventh Informational Supplement. CLSI Document M100-S27. *Clinical and Laboratory Standards Institute Wayne, PA.*
16. Lade, H., Paul, D., & Kweon, J. H. (2014). Isolation and molecular characterization of biofouling bacteria and profiling of quorum sensing signal molecules from membrane bioreactor activated sludge. *International Journal of Molecular Sciences, 15*(2), 2255–2273.
17. ICH Q2(R1) (2005). Validation of analytical procedures: text and methodology. *International Conference on Harmonization, Geneva*.
18. USP (2017). US Pharmacopeia National Formulary 2017: USP 27 NF 22. In (Vol. 2, pp. 2281). *United States Pharmacopeial Convention, Rockville*.
19. Yang, Y., Zhou, M., Hardwidge, P. R., Cui, H., & Zhu, G. (2018). Isolation and characterization of *N*-acyl homoserine lactone-producing bacteria from cattle rumen and swine intestines. *Frontiers in Cellular Infection Microbiology, 8*, 155.
20. Threlfall, E. J., Wain, J., Peters, T., Lane, C., De Pinna, E., Little, C. L., Wales, A. D., & Davies, R. H. (2014). Egg-borne infections of humans with *Salmonella*: not only an *S*. Enteritidis problem. *Worlds Poultry Science Journal, 70*(1), 15–26.
21. CDC. (2015). Centers for Disease Control National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): the 2013 NARMS annual human isolates report. *US Department of Health Human Services, CDC, Atlanta.*
22. Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Gast, R., Humphrey, T. J., & Van Immerseel, F. (2009). Mechanisms of egg contamination by *Salmonella* Enteritidis. *FEMS Microbiology Reviews, 33*(4), 718–738.
23. Khan, K. A., Khan, S. A., Aslam, A., Rabbani, M., & Tipu, M. Y. (2004). Factors contributing to yolk retention in poultry: a review. *Pakistan Veterinary Journal, 24*, 46–51.
24. Rehault-Godbert, S., Baron, F., Mignon-Grasteau, S., Labas, V., Gautier, M., Hincke, M. T., & Nys, Y. (2010). Effect of temperature and time of storage on protein stability and anti-*Salmonella* activity of egg white. *Journal Of Food Protection, 73*(9), 1604–1612.
25. Wyres, K. L., & Holt, K. E. (2018). *Klebsiella pneumoniae* as a key trafficker of drug resistance genes from environmental to clinically important bacteria. *Current Opinion in Microbiology, 45*, 131–139.
26. Struve, C., & Krogfelt, K. A. (2004). Pathogenic potential of *environmental Klebsiella pneumoniae* isolates. *Environmental Microbiology, 6*(6), 584–590.
27. Khelfa, D., & Morsy, E. A. (2015). Incidence and distribution of some aerobic bacterial agents associated with high chick mortality in some broiler flocks in Egypt. *Middle East Journal of Applied Sciences, 5*, 383–394.
28. Aly, M. M., Khalil, S., & Metwaly, A. (2014). Isolation and molecular identification of *Klebsiella* microbe isolated from chicks. *Alexandria Journal for Veterinary Sciences, 43*(1), 97–103.
29. Ojo, O. E., Ogunyinka, O. G., Agbaje, M., Okuboye, J. O., Kehinde, O. O., & Oyekunle, M. A. (2012). Antibiogram of Enterobacteriaceae isolated from free-range chickens in Abeokuta, Nigeria. *Veterinarski Arhiv, 82*(6), 577–589.

30. Ebomah, K. E., & Okoh, A. I. (2020). Detection of carbapenem-resistance genes in *Klebsiella* species recovered from selected environmental niches in the Eastern Cape Province, South Africa. *Antibiotics, 9*(7), 425.
31. Samanta, A., Mahanti, A., Chatterjee, S., Joardar, S. N., Bandyopadhyay, S., Sar, T. K., & Samanta, I. (2018). Pig farm environment as a source of beta-lactamase or AmpC-producing *Klebsiella pneumoniae* and *Escherichia coli*. *Annals of Microbiology, 68*(11), 781–791.
32. Wu, H., Wang, M., Liu, Y., Wang, X., Wang, Y., Lu, J., & Xu, H. (2016). Characterization of antimicrobial resistance in *Klebsiella* species isolated from chicken broilers. *International Journal of Food Microbiology, 232*, 95–102.
33. Kim, S.-H., Wei, C.-I., Tzou, Y.-M., & An, H. (2005). Multidrug-resistant *Klebsiella pneumoniae* isolated from farm environments and retail products in Oklahoma. *Journal of Food Protection, 68*(10), 2022–2029.
34. Wu, H., Liu, B., Liu, J., Pan, Y., Yuan, L., & Hu, G. (2012). Phenotypic and molecular characterization of CTX-M-14 extended-spectrum beta-lactamase and plasmid-mediated ACT-like AmpC beta-lactamase produced by *Klebsiella pneumoniae* isolates from chickens in Henan Province, China. *Genetics Molecular Research, 11*(3), 3357–3364.
35. Winokur, P., Canton, R., Casellas, J.-M., & Legakis, N. (2001). Variations in the prevalence of strains expressing an extended-spectrum β-lactamase phenotype and characterization of isolates from Europe, the Americas, and the Western Pacific region. *Clinical Infectious Diseases, 32*(Supplement_2), S94–S103.
36. Abdallah, N. M. A., Elsayed, S. B., Mostafa, M. M. Y., & El-gohary, G. M. (2011). Biofilm forming bacteria isolated from urinary tract infection, relation to catheterization and susceptibility to antibiotics. *International Journal of Biotechnology Molecular Biology Research, 2*(10), 172–178.
37. Savas, L., Guvel, S., Onlen, Y., Savas, N., & Duran, N. (2006). Nosocomial urinary tract infections: micro-organisms, antibiotic sensitivities and risk factors. *West Indian Medical Journal, 55*(3), 188.
38. Poonguzhali, S., Madhaiyan, M., & Sa, T. (2007). Production of acyl-homoserine lactone quorum-sensing signals is wide-spread in gram-negative Methylobacterium. *Journal of Microbiology Biotechnology, 17*(2), 226–233.
39. Wang, L.-H., Weng, L.-X., Dong, Y.-H., & Zhang, L.-H. (2004). Specificity and enzyme kinetics of the quorum-quenching *N*-acyl homoserine lactone lactonase (AHL-lactonase). *Journal of Biological Chemistry, 279*(14), 13645–13651.
40. Mohammed Sakr, M., Mohamed Anwar Aboshanab, K., Mabrouk Aboulwafa, M., & Abdel-Haleem Hassouna, N. (2013). Characterization and complete sequence of lactonase enzyme from *Bacillus weihenstephanensis* isolate P65 with potential activity against acyl homoserine lactone signal molecules. *BioMed Research International, 2013*, 1–10.
41. Alshammari, T. M., Al-Hassan, A. A., Hadda, T. B., & Aljofan, M. (2015). Comparison of different serum sample extraction methods and their suitability for mass spectrometry analysis. *Saudi Pharmaceutical Journal, 23*(6), 689–697.
42. See-Too, W. S., Convey, P., Pearce, D. A., & Chan, K.-G. (2018). Characterization of a novel *N*-acylhomoserine lactonase, AidP, from Antarctic *Planococcus* sp. *Microbial Cell Factories, 17*(1), 179.
43. Vega, L. M., Mathieu, J., Yang, Y., Pyle, B. H., McLean, R. J., & Alvarez, P. J. (2014). Nickel and cadmium ions inhibit quorum sensing and biofilm formation without affecting viability in *Burkholderia multivorans*. *International Biodeterioration Biodegradation, 91*, 82–87.
44. Ortori, C. A., Dubern, J.-F., Chhabra, S. R., Cámara, M., Hardie, K., Williams, P., & Barrett, D. A. (2011). Simultaneous quantitative profiling of *N*-acyl-L-homoserine lactone and 2-alkyl-4 (1H)-quinolone families of quorum-sensing signaling molecules using LC-MS/MS. *Analytical Bioanalytical Chemistry, 399*(2), 839–850.
45. Yates, E. A., Philipp, B., Buckley, C., Atkinson, S., Chhabra, S. R., Sockett, R. E., Goldner, M., Dessaux, Y., Cámara, M., Smith, H., & Williams, P. (2002). *N*-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infection Immunity, 70*(10), 5635–5646.
46. Leipert, J., Treitz, C., Leippe, M., & Tholey, A. (2017). Identification and quantification of *N*-acyl homoserine lactones involved in bacterial communication by small-scale synthesis of internal standards and matrix-assisted laser desorption/ionization mass spectrometry. *Journal of The American Society for Mass Spectrometry, 28*(12), 2538–2547.
47. Wang, J., Quan, C., Wang, X., Zhao, P., & Fan, S. (2011). Extraction, purification and identification of bacterial signal molecules based on *N*-acyl homoserine lactones. *Microbial Biotechnology, 4*(4), 479–490.
48. Fekete, A., Frommberger, M., Rothballer, M., Li, X., Englmann, M., Fekete, J., Hartmann, A., Eberl, L., & Schmitt-Kopplin, P. (2007). Identification of bacterial *N*-acylhomoserine lactones (AHLs) with a combination of ultra-performance liquid chromatography (UPLC), ultra-high-resolution mass spectrometry, and in-situ biosensors. *Analytical Bioanalytical Chemistry, 387*(2), 455–467.

49. Cruz, E., Euerby, M., Johnson, C., & Hackett, C. (1997). Chromatographic classification of commercially available reverse-phase HPLC columns. *Chromatographia, 44*(3-4), 151–161.
50. Wang, H., Cai, T., Weng, M., Zhou, J., Cao, H., Zhong, Z., & Zhu, J. (2006). Conditional production of acyl-homoserine lactone-type quorum-sensing signals in clinical isolates of enterobacteria. *Journal of Medical Microbiology, 55*(12), 1751–1753.