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Original article

ROLE OF OUTER MEMBRANE VESICLES OF HYPERVIRULENT *KLEBSIELLA PNEUMONIAE* ISOLATED FROM BOVINE UTERINE DISCHARGE IN PRO-INFLAMMATORY DYSREGULATION

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Summary

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Numerous research studies have established a connection between hypervirulent *Klebsiella pneumoniae* (hvKP) and bovine abortion, as well as other complications and clinical illnesses. These bacteria, which belong to the Gram-negative group, release outer membrane vesicles (OMVs), believed to play a crucial role in transporting effector molecules to their target cells. To investigate this further, OMVs of hvKP were collected from 47 clinical isolates from bovine uterine discharge samples using ultracentrifugation. Confirmation of the 47 isolates was carried out through a standard PCR test targeting the *16S rRNA* gene. Subsequently, a multiplex PCR was used to verify positive *16S rRNA* gene results by detecting specific virulence genes (*fimH, acrAB, traT,* and *rmpA*). The standard PCR test showed that 27 out of the 47 isolates (57.45%) tested positive for the *16S rRNA* gene, with all 27 of these isolates also testing positive for the *rmpA* and *traT* genes. Additionally, 30% of the isolates (8.1 out of 27) tested positive for *AcrAB*, while 70% of the isolates (18.9 out of 27) tested positive for *FimH*. During SDS-Western blotting, ten protein bands with molecular weights ranging from 18 to >60 kDa were identified in *K. pneumoniae*-derived OMV preparations. These bands included *OmpX*, *flagellin*, *MipA*, *OmpA*, *OmpC*, and *OmpF* (42–44 kDa), a strong *OmpA* band at 45 KDa, and a faint band at 60 kDa of *GroEL* as an oligomer of *OmpA*. Furthermore, hvKP OMVs displayed varying cytotoxic effects on the L929 and THP-1 cell lines. Notably, incubating THP-1 cells with high concentrations of hvKP OMVs inhibited IL-1β and TNF responses, indicating that these OMVs may not only facilitate the internalisation of the bacterium by THP-1 cells but also suppress the cells' innate immune response to hvKP infection. This ability of hvKP OMVs to evade the immune system and modulate host responses contributes significantly to the bacterium's pathogenicity.

Key words: bovine uterine discharge, hvKP, OMVs, pro-inflammatory response, SDS-Western blotting, virulence genes

INTRODUCTION

Over the past several decades, the frequency of illnesses caused by hypervirulent *Klebsiella pneumoniae* (hvKP) has progressively increased worldwide (Russo & Marr, 2019; Choby *et al.*, 2020; Ma *et al.*, 2023). The capacity to infect healthy people and animals of any age is one of the traits of the hvKP infection, which can cause several major clinical complications such as pneumonia, mastitis, and endometritis illness (Choby *et al.*, 2020; Darniati *et al.*, 2021). Even though the *Klebsiella pneumonia*e infection is often found in the urinary tract, the abortion process dilates the cervix and eliminates the cervical mucus plug, making it possible for vaginal pathogens to reach the uterus and infect the endometrium (Biksi, 2002; Aziz *et al.*, 2017). Increased vascular permeability at the site of an endometrial infection or from the presence of infected endometrial remnants can lead to systemic infection and septic shock. This case demonstrates the risk of septic shock syndrome following a medical abortion, even when antibiotics are used during the procedure (Kaponis *et al.*, 2012). The aetiology of the sickness produced by hvKP is poorly understood, despite the fact that this microbe poses a significant clinical illness.

Although hvKP's virulence is largely due to its capsular polysaccharide (CPS), it also contains *rmpA* and *magA*, both virulence genes associated with iron acquisition and aerobactin production (Choby *et al.*, 2020).

Gram-negative bacteria release outer membrane vesicles (OMVs) ranging in size from 10 to 300 nm in diameter at all stages of growth (Furuyama & Sircili, 2021). The outer membrane buds off, and the evaginated portion of the membrane is closed off just before the vesicle is released. This process results in a vesicle that is largely composed of outer membrane molecules but also contains periplasmic components like nucleic acids, proteins, and lipopolysaccharides (LPS) (Anand & Chaudhuri, 2016).

Certain pathogenic organisms may have virulence factors that are carried by OMVs (Jarzab *et al.*, 2020). OMV has been found in bacteria that are growing in culture media (Dell'Annunziata *et al.*, 2021), biofilms (Yonezawa *et al.*, 2009), *in vitro* infections (Carvalho *et al.*, 2019), and in the tissues of infected people or animals (Gorringe *et al.*, 2009). OMVs were discovered to be released from *Acinetobacter baumannii, Moraxella catarrhalis,* and *Neisseria meningitides* (Schaar *et al.*, 2013).

Vesicles isolated from *E. coli* O157:H7 have been hypothesised to play a role in the export of hazardous and genetic compounds (Van der Pol *et al.*, 2012; Zhang *et al.*, 2021). There have been a few recorded cases of OMV-host cell signalling pathways. Studies using *in vitro* cell lines showed that *Pseudomonas aeruginosa* OMVs induced an interleukin (IL)-8 response (Zhang *et al.*, 2021). In the same context, Lee *et al.* (2018) indicated that *K. pneumoniae* stimulates epithelial cells to continuously express proinflammatory cytokines like interleukin (IL-1 and IL-8).

There are 3000–10000 copies of the *traT* gene product in *E. coli* strains isolated from wild bacteria. This gene encodes a large OMV of 25,000 kDa. (Montenegro *et al.*, 1985). It is probably present as a lipoprotein and appears to be non-covalently bound to peptidoglycan on the outer membrane (Nikaido & Vaara, 1985). In addition to imparting host bacteria resistance to complement-mediated

opsonisation, the traT protein and the product of the neighbouring gene, *traS*, provide the surface exclusion feature (El Fertas-Aissani *et al.*, 2013).

A crucial virulence factor, *rmpA* (regular mucoid phenotype), causes capsule synthesis and hyperviscosity. Plasmid-localised virulence factor *rmpA* controls the high-mucus phenotype of hvKP (Ahmed & Alaa, 2016).

K. pneumoniae-derived OMVs are shown to play an important role in the microorganism-host interaction (Dell' Annunziata *et al.*, 2021), where they regulate miRNA genetic transcription and influence the host inflammatory response.

Cano *et al.* (2009) provided further evidence that *K. pneumoniae* capsular polysaccharide was linked to host cell cytotoxicity and that strains expressing varying quantities of capsule were not all equally virulent, also demonstrating that bacterial adhesion to host cells was not directly linked to the cytotoxicity of epithelial cells. These findings indicate that the capsular polysaccharide is not the only bacterial factor involved in *K. pneumoniae* pathogenesis, leading us to hypothesise that OMVs from *K. pneumoniae* might be the extracellular toxic complex identified by Joshi (2021).

In light of these intricate interactions and the potential implications for animal health, the primary objectives of this study were to identify the virulence genes present in *K. pneumoniae* isolated from bovine uterine discharge, to detect the protein components of OMVs, and to analyse their role in shaping the immune response within the host environment, using an *in vitro* model. This research holds the promise of shedding light on the mechanisms behind hvKP-related illnesses and could pave the way for novel strategies to combat these infections.

MATERIALS AND METHODS

Sample collection

This study was done at the Department of Obstetrics and Surgery, College of Veterinary Medicine, University of Al-Qadisiyah, from September 2022 to January 2023. During this time period, 47 samples of bovine uterine discharge were obtained using standard procedures (Brown, 1977) and quickly delivered to the lab for analysis. After overnight incubation at 37 ºC, the isolates were activated by culturing on CHROMagar Orientation. Then the isolates were subcultured on MacConkey agar and incubated for 24 hours at 37 °C. The appearance of pink, mucoid, lactose-fermenting colonies suggests that the isolates were *Klebsiella* spp., whereas the colonies on CHROMagar™ MH Orientation: Chromogenic Mueller Hinton Medium (Kanto Chemical Co., Inc., Japan) were big, spherical and had a metallic blue colour. Morphological examination revealed that *Klebsiella* colonies were enormous; on brain heart infusion agar colonies were dome-shaped; and on MaCconkey agar, colonies were mucoid lactose-fermenting.

Gram staining revealed the presence of Gram-negative bacteria in the form of short, fat, straight rods. To further illustrate the capsule, a negative stain was also conducted. The identification of *Klebsiella* species can be achieved by examining their metabolic activities, which include citrate utilisation, catalase production, oxidase activity, and indole formation (Sikarwar & Batra, 2011). The identified biochemical features encompassed catalase activity, citrate utilisation, urease production, lactic acid generation, a positive Voges-Proskauer (VP) test, and the ability to ferment various sugars, including glucose, lactose, sucrose, maltose, and

mannitol (Collee *et al.*, 1996; Winn Washington *et al.*, 2006).

Bacterial genomic DNA extraction

The ExiPrep™ Dx Bacteria Genomic DNA Kit (Bioneer, Republic of Korea) was used to extract genomic DNA from *K. pneumoniae* isolates. Bacteria cultivated overnight in BHI broth were pelleted in 1.5-mL microcentrifuge tubes by centrifugation at 10,000 rpm for one minute. The manufacturer's instructions were followed for genomic DNA extraction, which included adding suspension buffer to the bacterial cell pellets and retaining the resultant supernatant. Following that, the genomic DNA was analysed using a Nanodrop spectrophotometer and stored at –20 ºC until needed for the PCR experiment.

Multiplex polymerase chain reaction

A standard PCR test was employed to verify the existence of *Klebsiella pneumoniae* through the detection of *16S rRNA* (352 bp). Subsequently, the samples that showed positive results for *16S rRNA* underwent a multiplex PCR assay to evaluate the *rmpA*, *acrAB*, and *traT* genes, as per the earlier research findings (Table 1). The rationale behind incorporating these genes into this study aimed at enhancing the diagnosis of *Klebsiella pneumoniae*, should be noted. It is essential to bear in mind that the genes listed in Table 1, are not exclusive to *Klebsiella* bacteria alone; they encompass efflux pump genes and virulence genes.

The AccuPower® PCR PreMix kit (Bioneer, Korea) was utilised for the multiplex PCR test. Briefly, the master mix containing dNTPs 250M, Tris-HCl (pH 9.0), Taq DNA polymerase 1U, 10 mM, KCl 30 mM, stabiliser, and 1.5 mM $MgCl_2$ was used according to the kit's instructions to prepare a total volume of 20 µL by adding 5 µL of purified genomic DNA, 1.5 µL of each primers (forward and reverse), and 12 µL of AccuPower® PCR PreMix. The mixture was then transferred to the thermocycler to conduct the conventional PCR test according to the following protocol: an initial denaturation cycle at 95 °C for 5 min, followed by 30 amplification cycles consisting of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. Finally, a single cycle of the final extension step at 72 °C for 10 min concluded the process. The PCR results were analysed using 1% agarose gel. Further analysis including the SDS page and OMV purification, was accomplished using *Klebsiella pneumoniae* isolates from which the three genes were discovered.

Bacterial strains and OMVs purification

After routine and molecular testing, *Klebsiella pneumoniae* was detected, and purification of the OMVs was achieved from liquid cultures using a slight modification of the previously described method (Chutkan *et al.*, 2013; Li *et al.*, 2018). In a nutshell, bacterial cells were collected when they had reached the stationary phase after being subjected to a 16-hour aerobic shaking culture in lysogeny broth (LB) at 37 ºC. Subsequently, the harvested bacterial cells underwent double centrifugation at 23,800 g for 20 min at 4 ºC. After that, any remaining cells in the supernatants were filtered out using a 0.22 µm size filter (Millipore Corporation, USA). Using ultrafiltration technology with a 100 kDa, 50 mL filtration membrane, the supernatants were concentrated (Millipore, USA). Moreover, a pellet of OMVs was produced after the material was ultracentrifuged for three hours at 4 ºC and 40,000 rpm (Beckman Coulter, Germany).

Table 1. Primer design and product size for targeted PCR test and gene criteria of *Klebsiella pneumoniae*

Primers	Sequences	Association with *K. pneumoniae*	Type of gene	Size (bp)	Reference
16S rRNA	F: CGCGAAGAACCTTACCTGGT R: AGTTGCAGACTCCAATCCGG	Used for bacterial identification and phylogenetic analysis	Housekeeping gene	352	Esmaeel & Sadeq (2017); Mohammed *et al.* (2020)
fimH	F: GCCAACGTCTACGTTAACCTG R: ATATTTCACGGTGCCTGAAAA	Mediates adhesion to host tissues, which is important for colonisation.	Virulence-related gene	180	Rahmati *et al.*, 2022
traT	F: GGTGTGGTGCGATGAGCACAG R: CACGGTTCAGCCATCCCTGAG	Confers resistance to complement-mediated serum killing, related to virulence	Virulence-related gene	288	Cortés *et al.* (2002); El Fertas-Aissani *et al.* (2013)
acrAB	F: ATCAGCGGCCGGATTGGTAAA R: CGGGTTCGGGAAAATAGCGCG	Involved in antibiotic resistance, active extrusion of antibiotics	Antibiotic resistance gene	312	Rahmati *et al.* (2022)
rmpA	F: TGCAAACACGCAAAGGACAA R: AAGAGTGCTTTCACCCCCTC	Regulates capsule biosynthesis, a virulence factor	Virulence-related gene	835	Nedrow (2009); Esmaeel & Sadeq (2017)

By resuspending the OMVs in PBS, a quantitative Bradford test was carried out (Biotech, USA).

Western blot analysis of protein expression

Protein measurement. The Bradford method was used to determine the amount of protein in the samples. In this method, 1 mg/mL BSA bovine serum albumin was used as a standard. To prepare Bradford's reagent, 10 mg of Coomassie Blue G-250 was dissolved in 5 mL of 95% ethanol, and 10 mL of 85% phosphoric acid was added to it. Then the volume of the solution was increased to 100 mL. Concentrations of 0, 2, 4, 6, 10, 15, and 20 microliters of BSA standard and 20 microliters of samples were added to the wells of the 96-well plate in two repetitions. To each well, 40 microliters of Bradford's reagent were added, followed by pipetting 200 microliters of distilled water to each well, and incubation at room temperature for 5 minutes.

SDS-PAGE protocol. Glass moulds for SDS-PAGE were first properly washed and degreased with ethanol. Before preparing the gel and pouring it into the glass moulds, the two sides of the glass moulds were first fixed with clips, and the bottom of the moulds was insulated with paraffin to prevent the gel from leaking out. After adjusting the glass plates and making the lower separating gel, ammonium sulfate and N,N,N′,N′-tetramethylethylenediamine (TEMED) were added, and after mixing, they were slowly poured into the space between the glass plates. Immediately, 2 mL of butanol were slowly poured on the surface of the gel to prevent air penetration during the polymerisation of the gel and to smooth its surface. After gel polymerisation, butanol was washed off the gel surface using distilled water. Before pouring the upper stacking gel, ammonium sulfate and TEMED were added, and after mixing, it was slowly poured into the space between the glass plates. Then the separating shoulders were placed between the two windows.

Before performing electrophoresis, protein samples were mixed with 2× sample buffer (loading buffer) at a one-to-one ratio based on the concentrations obtained in the Bradford method and boiled for 5 minutes. In this situation, proteins become linear. Then, to remove the steam, a quick centrifugation for 5 s was performed and placing on ice to cause the vapours come down and make the solution uniform. After pouring the samples into the electrophoresis wells, the electric current was first established for 15 min with electric tension of 60 V and then for an hour with 100 V. In each electrophoresis run, one well was dedicated to the protein marker (ladder).

Western Blotting technique protocol. After conducting protein electrophoresis through the SDS-PAGE technique, the subsequent steps were performed as follows: The gel was immersed in the transfer buffer for a duration of 10 to 15 min. Next, a sandwich configuration comprising 5 layers was created, consisting of two sponge layers, two layers of Whatman paper on either side, one layer of polyvinylidene difluoride (PVDF) paper (Amersham, 0.45 µm, USA), and one layer of gel. Prior to the preparation, all substances listed were soaked in the transfection buffer for 15 min. After making sure that there were no bubbles in the gel, the sandwich with the cassette base was placed in the blotting tank that was already filled with the transfer buffer, so that the blotting paper was placed on the cathode side and the gel on the anode side. Then the transmission was done at

60 V for 105 min. After the transfer, the paper was rinsed three times with PBS for 5 min each time. Afterwards, blocking was done by blocking buffer for 2 hours in the refrigerator at a temperature of 4 ^{o}C. The PVDF paper was blocked, then washed three times for 5 min each time with PBS, and incubated with primary antibodies prepared with a dilution of 1:500 in PBS buffer for one hour at room temperature and on a shaker at 65 rpm. Blotting was done separately for each gene. After incubation, the paper was washed three times with PBS for 5 min each time. Incubation for secondary antibodies prepared with a 1:1000 dilution in PBS buffer was done for 1 hour. After incubation, the PVDF paper was washed three times with PBS for 5 minutes each time. The two solutions of the ECL kit (ABCAM, 133408, USA) were combined in a 1:1 ratio in the amount of 250 microliters, and poured on the PVDF paper with the help of sampler 1000, and the paper was soaked with it for 1 minute. All processes were performed in a dark room. After leaving the emergence solution, the papers were dried in the environment, then were placed in a plastic protective cassette (RPI, USA) containing autoradiography-sensitive film (BioBlue-Lite™, USA), and bands are developed and analysed in a quantitative luminescence processor (ChemiProXS, Cleaver Scientific, England). Finally, to quantify the bands, the density of each protein relative to the calibrator protein in the studied groups was compared to the density of the target protein relative to the calibrator protein in the control group.

Cell culture

THP-1 and L929 cell lines were cultivated in Dulbecco's modified Eagle medium (DMEM; Invitrogen). In addition, 10% foetal bovine serum (FBS) and 100 units/mL penicillin were included. Each cell line was grown in an incubator at 37 ^{o}C with 5% carbon dioxide. All cells were thought to be in better cellular growth conditions (Cano *et al.*, 2009).

Cell proliferation assay

The proliferation of cells treated with increasing concentrations of OMVs was assessed using a CCK8-kit (Dojindo, Japan) assay to establish whether OMVs had a cytotoxic effect on cell viability. Each cell type was implanted in a 96-well microplate at a concentration of 5.0×10^4 cells (100 µL). Next, KP OMVs were titrated against adherent cells over the course of 24 hours, with each concentration being used once. Following the addition of CCK8, infected plates were kept in a humidified incubator at 37 ^{o}C and 5% carbon dioxide for 2 to 4 hours. Optical density (OD) readings at 450 nm were then used to track cell proliferation and viability. Following the completion of all tests in triplicate, the cell viability percentage (%) was calculated as $[(OD450_{sample}-OD450_{blank}) / [(OD450_{control}-OD450_{blank})]\times100$

ELISA

To determine the OMVs' most likely intended function, THP-1 cell lines were incubated with KP OMVs for 24 hours at 37°C in the presence of 5% FBS after being implanted at a density of 1×10^5 cells/well. The protein concentration of OMVs was significantly different, ranging from 0 µg/mL to 10 µg/mL. Pro-inflammatory cytokines IL-1β and TNF in the cell culture supernatants were quantified using an ELISA kit (BT LAB, China).

Statistical analysis

The data are presented as the mean ± standard deviation for triplicate experiments. Origin Lab software was utilising for statistical assessments and GraphPad Prism for drawing figures, while ANOVA was employed to compare the samples.

RESULTS

16S rRNA gene detection

In the standard PCR examination, 27 of the 47 isolates (57.45%) exhibited a positive *16S rRNA* gene response (Fig. 1A).

Multiplex PCR for rampA and traT

For the 27 isolates carrying the *16S rRNA* gene, multiplex PCR was utilised to identify two virulence-related genes: *rmpA* and *traT*. Notably, all 27 isolates yielded positive results for *rmpA* (835 bp) and *traT* (288 bp) (Fig. 1B).

Furthermore, the multiplex PCR examination targeting the *acrAB* and *fimH* genes, carried out on the same set of 27 isolates that had tested positive for the *16S rRNA* gene, yielded noteworthy results. Specifically, 30% of these isolates (8.1 out of 27) displayed positivity for *acrAB*, characterised by a fragment size of 312 bp. Additionally, 70% of the isolates (18.9 out of 27) tested positive for *fimH*, with a fragment size measuring 180 bp (Fig. 1B).

OMVs characterisation and separation

The OMV production of *K. pneumoniae* was evaluated through growing on a Petri dish. To obtain OMVs, the supernatants of bacteria were processed through an ultracentrifugation system. Vesicles with sizes of less than 100 nm were used to identify purified OMVs (Fig. 2).

KP OMVs separated by SDS-PAGE were found to contain numerous protein bands (Fig. 3A). Upon conducting Western blotting analysis, the purified OMV preparations derived from *K. pneumoniae* exhibited approximately 10 prominent protein bands with molecular weights (MW) from 18 to >60 kDa (Fig. 3B).

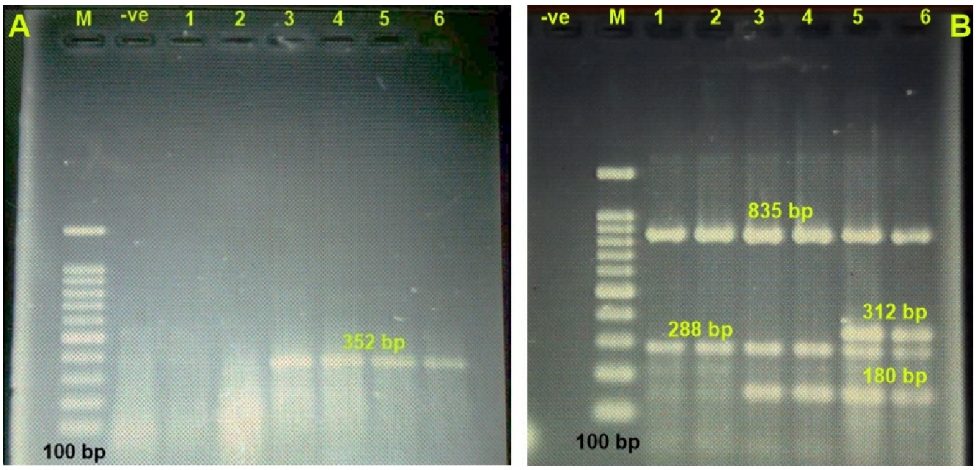

Fig. 1. PCR analysis results, showing positive amplification for the *16s rRNA* gene (352 bp) (A) and the *rmpA* (835 bp), *acrAB* (312 bp), *traT* (288 bp), and *fimH* genes (180 bp) (B). Lanes 1–6 represent the tested samples, -ve: negative control; M: the 100 bp ladder.

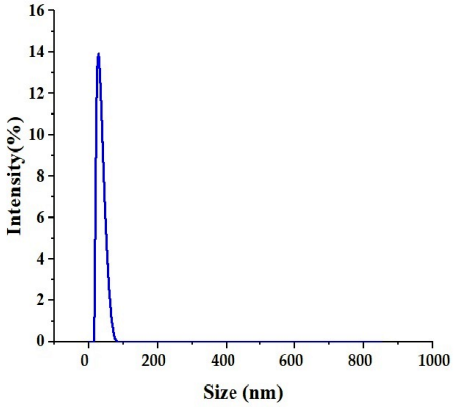

Fig. 2. Size of OMVs derived from *K. pneumoniae* using ultracentrifugation system.

The estimated MWs of these protein bands were 18, 29, 31, 35, 35–45 (three closely aligned protein bands), and 42 kDa. The identified protein bands corresponded to *OmpX*, *flagellin*, *MipA*, *OmpA*, *OmpC*, and *OmpF* (42–44 kDa), a single strong band at 45 KDa of *OmpA*, and a weak band at 60 kDa of *GroEL* as an *OmpA* oligomer. The data from the SDS-Western blotting analysis are summarised in Table 2.

OMVs cytotoxicity

To test the hypothesis that the variety of proteins found in KP OMVs may be responsible for oxidative stress or cytotoxicity in the host during *in vitro* or *in vivo* infection, the L929 and THP-1 cell lines were treated with varying concentrations of OMVs to determine whether OMVs supported the proliferation of host cells. Notably, cellular growth suppression was detected in both cell lines (L9292 and THP-1), although treating with OMVs at high concentrations 1–1.4 µg/mL exhibited stronger growth inhibition (Fig. 4).

In vitro estimation of OMVs' proinflammatory response

After 24 hours of exposure to OMVs, proinflammatory cytokine stimulation was assessed in THP-1 cells using ELISA. Our

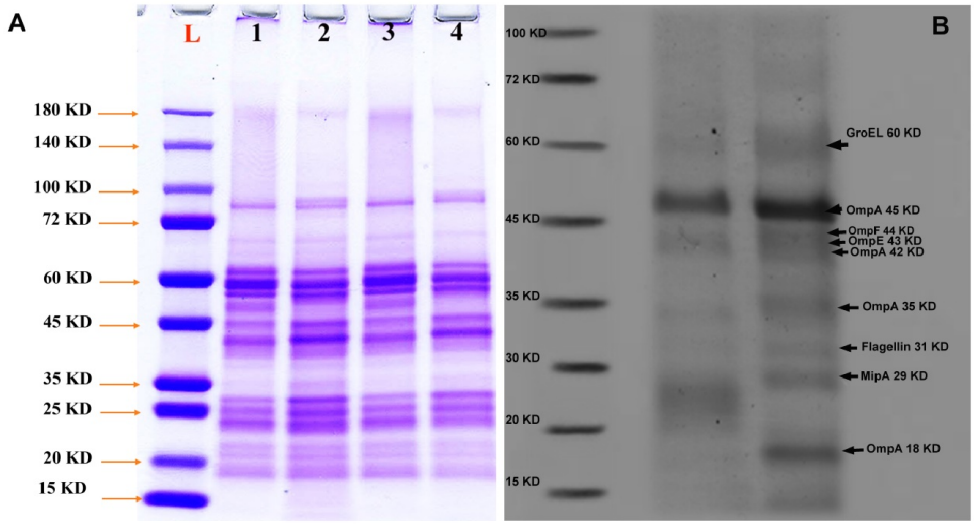

Fig. 3. The production of outer membrane vesicles (OMVs) by *Klebsiella pneumoniae* in an *in vitro* culture. **A.** OMV proteins from *K. pneumoniae* separated using a 12% SDS-PAGE concentration; **B.** Western Blotting – lane L: the ladder, lanes 1–4: OMV proteins.

Table 2. SDS-Western Blotting analysis of the outer membrane vesicles (OMVs) from *Klebsiella pneumoniae*

Protein size (kDa)	Antibody used	Band intensity	Band range (kDa)	Identified protein
15–20	Anti-OmpX	strong	18	Outer membrane protein X
20–30	Anti-MipA	moderate	29	MltA-interacting protein
30–35	Anti-Flagellin	weak	31	Lipoprotein
35–45	Anti-OmpA	moderate	35	Outer membrane protein A
35–45	Anti-OmpA	moderate	42–44	Outer membrane protein A, C, F
40–45	Anti-OmpA	strong	42	Outer membrane protein A
45–60	Anti-OmpA	weak	60	An oligomer of OmpA

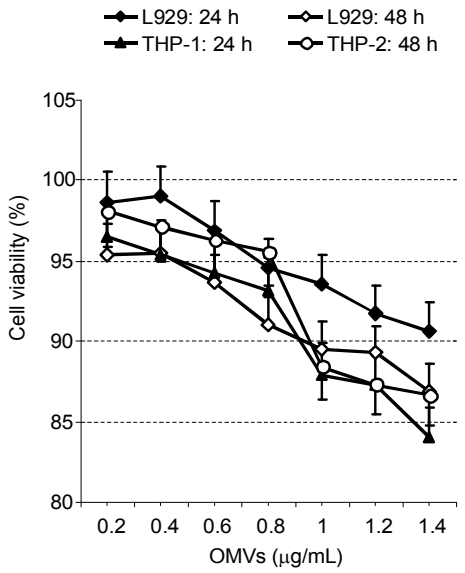

Fig. 4. Cytotoxicity (%) of different hvKP OMVs concentrations (μg/mL) on L929 and THP-1 cells.

findings indicated that OMVs originating from hvKP disrupted the release of IL-1β and TNF from THP-1 cells in a dose-dependent manner, with a decrease in IL-1β and TNF levels in pg/mL proportionally to the increased concentration of OMVs. The preincubation of cells with 0.1, 1, and 10 μg/mL of OMVs inhibited the IL-1β response by 80 pg/mL, 60 pg/mL and 40 pg/mL, respectively (Fig. 5A). In the case of TNF (Fig. 5B), inhibition concentrations were 150 pg/mL, 120 pg/mL and 80 pg/mL, respectively.

DISCUSSION

The *rmpA* gene was chosen because it is one of the virulence genes of *Klebsiella* bacteria, and genetic identification of *rmpA* (Yu *et al.*, 2006) appears to be more successful at increasing the detection rate of hvKP. Additionally, distinct *Enterobacteriaceae* species isolates show resistance to complement bacterial lysis activity when *traT* genes are present, which encode outer membrane proteins (OMVP) and increased serum survival (*iss*) gene (Agüero *et al.*, 1984).

The well-studied pathway for making OMV seems to be the same in all Gram-negative bacteria (Badouei *et al.*, 2016). Several *Enterobacteriaceae* species, such as *E. coli*, *S. flexneri*, *S.* Typhimurium, *K. pneumoniae*, *S. marcescens*, *P. vulgaris*, *P. mirabilis*, and *P. stuartii*, are recognised for possessing an OMP gene cluster. In the tested strains, each species displayed at least one significant outer membrane protein associated with peptidoglycan, with the majority of them

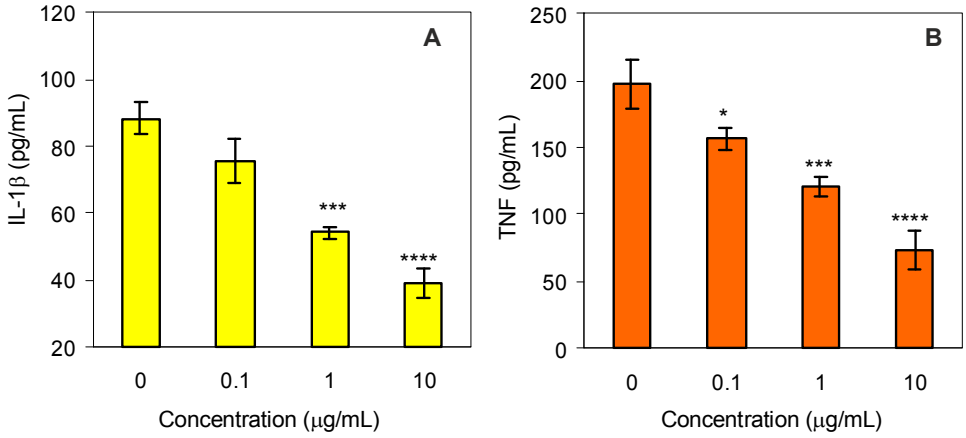

Fig. 5. *In vitro* THP-1 and cells activity of hvKP OMVs on induction of proinflammatory response following a 24-hour incubation period: A. IL1-β; B. TNF; Data are presented as the mean±SD of triplicate experiments; *P<0.05; ***P<0.001; ****P<0.0001 vs 0 μg/mL.

harbouring multiple such proteins (Kulp & Kuehn, 2010).

According to the current data and a previous study that detected the prevalence of *traT* in Gram-negative bacteria, the *traT* gene has been found to be more common in *E. coli* strains isolated from individuals with gastrointestinal and urinary tract infections (Kulp & Kuehn, 2010).

Our results demonstrated that during *in vitro* cultivation, KP created OMVs spontaneously and secreted them into the extracellular milieu in a way similar to that of other OMV-producing organisms. Several proteomics analyses of OMVs have yielded the surprising finding that 277 proteins can be pinned down as components of OMVs secreted by *Acinetobacter baumannii* strains. The majority of these proteins have been shown to exhibit high levels of stress and survival proteins (Bhar *et al.*, 2021). Proteins are packed into OMVs by *Klebsiella pneumoniae* preferentially, as shown by prior biochemical and proteomic studies (Khan *et al.*, 2017). *Mycobacterium* TB vesicles were shown to include a variety of proteins and that several of these proteins were involved in bacterial virulence (Cahill *et al.*, 2015).

Our findings indicated that KP OMVs mostly contained cytoplasmic and ribosomal proteins in addition to the *RcsF* and *OmpA* family lipoproteins (Zhang *et al.*, 2021). Antibiotic susceptibility is increased in the absence of outer membrane proteins (OMPs), which are essential for antibiotic diffusion and membrane permeability. Evidence of OMPA expression in the presence of OMVs suggests it may aid in resistance to antibiotics (Brown *et al.*, 2015).

In this research, hvKP OMVs not only inhibited the proliferation of L929 and THP-1 cells but also induced cell death in some instances, indicating a potential cytotoxic impact. It is interesting to note that *Pseudomonas aeruginosa* OMVs, as well as *Porphyromonas gingivalis* OMVs, have been linked to host cell cytotoxicity (Walsh *et al.*, 2003). Furthermore, apoptotic cell death was found to be the primary result of co-incubation of epithelial

cells with spontaneously released OMVs from *Stenotrophomonas maltophilia* (Nakao *et al.*, 2014). OMVs from *Klebsiella pneumoniae* contain many proteins, lipopolysaccharides (LPS), and peptidoglycans, all of which may cause interaction with host cells and a change in host cell biology (Gomes *et al.*, 2021).

Previous studies on *K. pneumoniae*-induced host cell disease showed a correlation between the cytotoxicity of these cells and the extracellular components produced or released by the bacteria (Zhang *et al.*, 2021; Al-Musawi *et al.*, 2022). As a result, we hypothesised that *K. pneumoniae* OMVs would stunt the proliferation of L929 cells and THP-1 cells. Several other research investigations (Lee *et al.,* 2012; Li *et al.,* 2023) contradicted our findings by indicating that OMVs from *K. pneumoniae* (specifically, KP ATCC 13883) had no cytotoxic effects on either cell type. Furthermore, these OMVs did not inhibit cell growth, and the proteome study of *K. pneumoniae* OMVs did not detect any cytotoxic components (Lee *et al.*, 2012). However, it is yet to be established if OMVs from other strains of *K. pneumoniae* are harmful to host cells.

OMVs from *K. pneumoniae* caused proinflammatory responses *in vitro*. The expression of the proinflammatory cytokines IL-1β and TNF was slightly reduced after treatment with a high dose of *K. pneumoniae* OMVs, which could be due to the cytotoxicity of OMVs on THP-1 cells. These findings suggest that *K. pneumoniae* OMVs, like other Gram-negative bacteria, such as *Salmonella enterica* serovar Typhimurium and *Helicobacter pylori* (Lee *et al.*, 2012; Liu *et al.*, 2016), stimulated the expression of proinflammatory cytokine genes in THP-1 cells in a non-dose-dependent manner (Lee *et al.*, 2012).

OMVs are produced by pathogens and interact with neighbouring epithelial cells and immune cells to set off the immune system (Ismail *et al.*, 2003). Due to their small size, OMVs can transfer immunomodulation substances like protein, flagellins, peptidoglycans, lipids, and LPS over the mucosal barrier (Amano *et al.*, 2010). This dysregulation results from either preventing dendritic cell activation by altering the Akt-Nrf2 and mTOR-IKK-NF-B signalling pathways, which regulate dendritic cell formation and function, or inhibiting T cell activation by overexpressing IL-10 (Winter *et al.*, 2014).

In Gram-negative bacteria, these immune activating ligands interact with host cells to induce inflammatory responses (Furuyama & Sircili, 2021). Outer membrane vesicles (OMVs) are released by many pathogens and can either encourage or discourage the recruitment of inflammatory cells to the surface of mucosal epithelial cells (Lee *et al.*, 2012). OMVs can interact directly with epithelial cells or pattern recognition receptors (TLR-4), resulting in either an increase or inhibition production of cytokines and chemokines. Tight junction disruption can also result from interactions between epithelial cells and OMVs, allowing OMVs and their bacterial virulence factors to penetrate the submucosa and engage with neutrophils, dendritic cells, and macrophages (Cahill *et al.*, 2015) .

According to studies, IL-1β and IL-8 are proinflammatory cytokines that recruit inflammatory cells to the site of an infection (Lee *et al.*, 2018). The use of neutropenic animals mitigated the effects of *K. pneumoniae* OMVs on inflammatory cell infiltration in treated mice. In addition, Zhang *et al.* (2021) showed that *K. pneu-*

moniae OMVs do not serve as a vector for delivering noxious substances to host cell, but rather work as a powerful immune modulator to set off an inflammatory response. These findings expand the function of OMVs in the pathogenesis of the major opportunistic pathogen, *K. pneumoniae*.

CONCLUSION

In this work, hvKP was identified utilising a PCR assay with *16S rRNA* and 4 virulence genes: *fimH, acrAB, traT, and rmpA*. SDS-Western blotting showed that OMV prepared from *K. pneumoniae* had ten protein bands with sizes between 18 and >60 kDa. These bands included *OmpX*, *flagellin*, *MipA*, *OmpA*, *OmpC*, *OmpF*, and *GroEL* as an oligomer of *OmpA*. In addition, this *in vitro* investigation indicated that hvKP OMVs included various proteins with the ability to influence immune response pathways. This work assigned a role for OMVs in causing cytotoxicity during *in vitro* infection of L929 and THP-1 cell lines. The results showed that the OMVs derived from hvKP dysregulated the release of IL-1β and TNF from THP-1 cells in a dose-dependent manner. During hvKP infection, OMVs can interact directly with epithelial cells, allowing OMVs and their bacterial virulence factors to penetrate the submucosa and engage with neutrophils, dendritic cells, and macrophages, which may explain the pathogenesis of *K. pneumoniae*.

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