UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF BACTERIOPHAGE ΦKAZ14 LOADED CHITOSAN NANOPARTICLES FOR BIOLOGICAL CONTROL OF COLIBACILLOSIS IN CHICKENS

ADAMU AHMAD KAIKABO

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By

ADAMU AHMAD KAIKABO

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

June 2016

DEDICATION

To the

Memories of

My late mother Hauwa, elder and younger sisters Aisha and Salamatu Muhammad Kaikabo Adamu. May Allah bless their souls and admit them into al-jannatul Firdaus.

…and

My children

Adama (Hamra), Hajara (Khairat), Sumayya and AbdurRahman (Arafat)

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

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Chairman : Abdulkarim Sabo Mohammed, PhD
Faculty : Food Science and Technology

Bacteriophage ΦKAZ14 was applied in the biological control of colibacillosis. It had lytic activity on APEC 01: K1:H7 and β lactamase producing *Escherichia coli*. The icosahedral head of the phage measured 50 nm by 45 nm and long contractile tail of 78 nm by 10 nm. It multiplicity of infection (MOI) was 0.01, the latent period of infection of 15 minutes and burst size of 80 particles per infected cell. The phage survived a pH range of 5.0 to 14, and resistant to a temperature of 65 ℃. C-NPs protected the phage from enzymatic degradation *in vitro*. The spherical C-NPs had average particle size range from 176 ± 3.2 to 188 ± 7.4 nm, a zeta potential of 60.3 ±0.2 and 50.5 ±0.4 and polydispersity index (PDI) of 0.506 and 0.472. Fourier transform infrared spectroscopy (FTIR) showed no difference between C-ΦKAZ14 NP and C-NP. Thermal analysis showed the temperature of -20 ℃ is desirable for storage. C-ΦKAZ14 NPs were not toxic on chorioallantoic membrane. Treatment with C- ΦKAZ14 NPs decrease viable bacterial counts in feces and colonization in the intestine of chickens ($p \leq 0.05$). It also significantly decreased mortality rate in *E. coli*-challenged birds ($p \leq 0.05$). C- ΦKAZ14 NPs increased bodyweight of chickens ($p \leq 0.05$). Lesions were not observed in major organs. C-ΦKAZ14 NPs therapy is a beneficial control strategy against colibacillosis in chickens.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PEMBANGUNAN BAKTERIOFAJ ΦKAZ14 SARAT NANOPARTIKEL CHITOSAN BAGI KAWALAN BIOLOGI COLIBACILLOSIS PADA AYAM

Oleh

ADAMU AHMAD KAIKABO

Jun 2016

Pengerusi : Abdulkarim Sabo Mohammed, PhD.
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Bakteriofaj ΦKAZ14 telah digunakan dalam kawalan biologi colibacillosis. Ia mempunyai aktiviti pemusnahan pada APEC 01: K1: H7 dan *Escherichia coli* yang menghasilkan β lactamase. Kepala icosahedral daripada faj yang diukur 50 nm sebanyak 45 nm dan ekor panjang contractile 78 nm oleh 10 nm. Kepelbagaian jangkitan (MOI) adalah 0.01, tempoh pendam jangkitan 15 minit dan saiz pecah adalah 80 zarah bagi setiap sel dijangkiti. Faj terselamat pada julat pH 5.0 hingga 14, dan tahan suhu hingga 65 ℃. C-NPS melindungi faj dari kemusnahan enzim in vitro. C-NPs sfera mempunyai purata julat saiz zarah dari 176 ± 3.2 ke 188 ± 7.4 nm, potensi zeta 60.3 ± 0.2 dan 50.5 ± 0.4 dan indeks polikeselerakan (PDI) pada 0.506 dan 0.472. Spektroskopi inframerah ubah Fourier (FTIR) menunjukkan tiada perbezaan antara C-ΦKAZ14 NP dan C-NP. Analisis terma menunjukkan suhu -20 ° C adalah wajar untuk penyimpanan. C-ΦKAZ14 NPs adalah tidak toksik terhadap membran chorioallantoic. Rawatan dengan C- ΦKAZ14 NPS mengurangkan bilangan bakteria berdaya maju dalam najis dan pengkolonian dalam usus ayam ($p \leq 0.05$). Ia juga menurunkan kadar kematian dengan ketara dalam burung yang dijangkiti E. coli ($p \leq 0.05$). C- ΦKAZ14 NPs meningkatkan berat badan ayam ($p \leq 0.05$). Luka-luka tidak didapati dalam organ-organ utama. Terapi C- ΦKAZ14 NPs adalah strategi kawalan bermanfaat menentang colibacillosis pada ayam.

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I certify that a Thesis Examination Committee has met on 14 June 2016 to conduct the final examination of Adamu Ahmad Kaikabo on his thesis entitled "Development of Bacteriophage ΦKAZ14-Loaded Chitosan Nanoparticles for Biological Control of Colibacillosis in Chickens" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

APEC	Avian pathogenic *Escherichia coli*
bp	Base pair
cP	Centipoise
CE	Communautés européennes
CAM	Chorioallantoic membrane assay
CHROMagar	Chromogenic agar
CNF	Chicken normal flora
C-NPs	Chitosan nanoparticles
C- ΦKAZ14	Chitosan phi kappa alpha zeta 14
CFU/mL	Colony forming unit per milliliter
ColV	Colcin V
DAL	Double agar layer
DSC	Differential scanning calorimetry
ds	double stranded
EC	European commission
EHEC	Enterohaemorragic *Escherichia coli*
Et	Endotoxin
ETEC	Enterotoxinogenic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESBL	Extended spectrum beta lactamase
EU	European Union
FRD1	*Pseudomonas aeruginosa* strain
FTIR	Fourier transform infrared resonance
GC	Gas chromatography
GC-MS	Gas Chromatography Mass Spectrometry
HACCP	Hazard Analysis Critical Control Point
hCG	Human chorionic gonadotropin
HCN	Hydrogen cyanide

HUS	Hemolytic uremic syndrome
^{1}H NMR	Proton Nuclear Magnetic Resonance
IFPRI	International Food Policy Research Institute
IL-6	Interleukin 6
kDA	Kilo Dalton
LBA	Luria Bertani Agar
LBB	Luria Bertani Broth
LPS	Lipopolysaccharide
LyD	Lysis deficient
L-glutamic acid	L-form of Glutamic acid
MS	Mass spectrometry
MRSA	Methicillin resistant *Staphylococus aureaus*
MSSA	Methicillin sensitive *Staphylococcus aureaus*
M13 phage	M13 Bacteriophage
NEM	Newborn *Escherichia coli* Meningitis
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NPs	Nanoparticles
PCR	Polymerase chain reaction
PDI	Polydispersity index
PFU/mL	Plaque forming unit per milliliter
PlyG	Phage lysin-Gamma
pH	Measure of acid base concentration
RES	Reticulo Endothelial System
RNA	Ribonucleic acid
rpm	Revolution per minute
16S rRNA	16S ribosomal RNA
SEM	Scanning electron microscopy
T lymphocytes	Thymus derived lymphocytes
Th 1	Thymus helper cells 1

Th 2	Thymus helper cells 2
TEM	Transmission electron microscopy
TMP-STX	Trimethoprim-Sulfamethoxazole
TNF	Tissue necrosis factor alpha
TPP	Tripolyphosphate
UTI	Urinary Tract Infection
v/v	Volume per volume
µm	Micrometer
µL	Microliter
%	Percentage
ΦKAZ14	Phi Kaikabo Azbak 14

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Poultry sector is an important component of the livestock industry in Peninsular Malaysia and the world in general. This sector has metamorphosed into a progressive, coordinated, and advanced industry with egg and meat production worth between RM1.78 to RM6.03 billion annually. The global poultry meat consumption had increased from 66 million tonnes in 2000 to a calculated 106 million tonnes in 2013, and of this increase, nearly 40 % of the consumption rates were in Asia. Malaysia is self-reliant in the production of poultry meat (128%) and eggs (115%) required for its growing population (Jamaluddin, 2013). The poultry Subsector is the most adjustable and rapidly growing of the livestock sector. Poultry represents a substantial portion of food animal and contributes significantly to economic growth and food security globally (FAO, 2013). The annual chicken meat consumption per person will increase by 26% (FAO, 2010). An estimated 40% of the total world animal protein is contributed by the poultry industries in the year 2015 (IFPRI, 2000).

Malaysia even though is self-reliant in poultry meat and egg production have pertinent issues with regard to production and sustainability. As the poultry industry is expanding, the need to improve the efficiency of production will largely depend on the control and prevention of diseases. Infectious diseases are one of the major causes of economic losses in poultry industries which are also a food safety issue. Rapid growth in this industry is only possible with innovative advances in poultry management, nutrition, genetics and better diagnosis and control of diseases (Bagust, 2013).

On the global perspective, poultry production has been projected to increase by an unprecedented 119.4 million metric tonnes from the year 2012 to 2030, an increase of 31.6%. Among the top poultry producing countries, India is expected to top the list at 102.1%, Russia 45.2%, Brazil 40.1%, China 33.4%, United State 24.5%, and the European Union 7.4%. However, the trend is expected to decline by 0.7% from 733,000 to 728,000 metric tonnes chicken meat production in Egypt and a drop of 6.1% from 578,000 to 543,000 metric tonnes in Taiwan (USDA ERS, 2015).

Diseases of bacterial and viral origin are responsible for the decline in the growth of the poultry industry. Bacterial disease such as colibacillosis causes huge economic losses to the poultry industry worldwide (Youssef & Mansour, 2008). It is a major infectious disease observed in birds of all ages, thus regarded as one of the commonest infectious diseases of poultry that causes morbidity and mortality in young chicks (Li et al., 2005).

Practical strategies for the control of Colibacillosis besides biosecurity management are; chemotherapy using antibiotics or vaccination as preventive measures against infection.

However, control by antibiotics is largely compromised by the emergence and development of resistant strains to one or more antibiotics drugs of choice (Vandekerchove et al., 2005). Also, in some cases, the reappearance of colibacillosis outbreak does occur following stoppage of the antibiotic chemotherapy (Zanella et al., 2000). The possibility of transfer of resistance genes from animal to human bacteria strains has been observed, and this is of great concern to public health.

Vaccination as a control strategy is not without practical issues. Literature has shown that APEC strains are highly diverse. This diversity has limited the applicability of vaccine even with the use of a genetically produced vaccine. Killed or attenuated vaccines provide sufficient protection against infection with homologous strains only, but protection against heterologous strains is with limited success (Dho-moulin et al., 1999). The compounding effects of lack of vaccine protection against infections on the production, economy and food safety and security are enormous.

Alternatively, control of Colibacillosis may be achieved through biological control strategy through the use of Bacteriophages. Phage is viruses that kill bacteria, they are specific to pathogens. A bacteriophage is not harmful to plants, human, animals or the environment, thus offer a useful alternative for the control of colibacillosis. Bacteriophages are capable of infecting and killing target bacteria and thus an alternative to combating antibiotic resistance The usefulness of Bacteriophage in control of Colibacillosis could be enhanced by encapsulation in materials like chitosan, which is a biodegradable biocompatible, nontoxic polymer which, when used in the encapsulation of a phage would protect and improve its delivery to the target sites of infection. This technology considered in this study.

1.2 Problem statement

Avian Pathogenic *Escherichia coli* is of potential concern to public health due the evidence of its possible role in causing human disease. It has been shown that *Escherichia coli* O2:K1 and O78 serotype can be isolated from infected human urinary tract and septicemic chickens.

Avian Pathogenic *Escherichia coli* colonizes gastrointestinal tract of chicken and this sometimes contributes to carcass contamination at slaughter and environmental pollution due to the fecal shedding of the organisms.

1.3 Significance of the study

Public health concerns arising from food safety are a burning issue in global trade of animal products and poultry in particular. In order to mitigate food safety risks; animal-derived food products, especially of poultry origin must be safe for human consumption. The strategy of using encapsulated or loaded bacteriophage in nanoparticles as a biocontrol agent could lead to the reduction in intestinal colonization of APEC and shedding into the environment, and would significantly reduce carcass contamination at

the slaughter.

1.4 Objectives of the study

The main objective of the current study was to prepare coliphage loaded in chitosan nanoparticles for the biocontrol of colibacillosis in chickens, and the specific objectives are:

1. To isolate and characterized lytic bacteriophage for the biological control of APEC O1:K1:H7
2. To prepare and characterized chitosan nanoparticle for the encapsulation of bacteriophage lytic to APEC O1:K1:H7
3. To evaluate in vivo efficacy of bacteriophage(s) encapsulated in chitosan nanoparticles for the biological control of APEC O1:K1:H7 in Chickens.

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