Quantitative detection and inactivation of *Mycoplasma hyopneumoniae*

By

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Submitted in fulfilment of the academic requirements for the degree of Master of Science in Biochemistry,

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2022

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Preface

The data presented in this dissertation was collected at the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, Republic of South Africa from 2021 to 2022. Experimental work was carried out at JASS from 2019-2021, under the supervision of Dr. Qiyan Xiong, while registered at the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Dr. Thandeka Khoza.

This dissertation submitted for the degree of Master of Science in the College of Agriculture, Engineering and Science, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, represents original work by the author and has not been submitted in any form for a degree or diploma at any other university.

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January 2023

I certify that the above statement is correct and as the candidate's supervisor I have approved this dissertation for submission.

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Dr. Thandeka Khoza (Supervisor) Dr. Qiyan Xiong (Co-supervisor)

08 February 2023 08 February 2023

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I, Yanna Wei (220102940), declare that:

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Declaration - Publications

Journal of Immunological Methods, research paper, Available online 26 November 2021

Development of an indirect competitive enzyme linked immunosorbent assay for the quantitative detection of *Mycoplasma hyopneumoniae* during the vaccine production process

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Authors' contributions

YW established and optimized the ic-ELISA assay and prepared the manuscript. Application of the ic-ELISA in the vaccine manufacturing process was carried out by TK. YY prepared the recombinant protein and polyclonal antibodies. LW, BL, and JW helped with the CCU assay. LG helped with the flow cytometry analysis. FH and GS modified the manuscript. QX and ZF supervised and guided this work. All authors read and approved the final manuscript.

Student: [redacted] Date: 08 February 2023

Supervisor: [redacted] Date: 08 February 2023

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Vaccine, prepared for under review

Comparative study on the inactivation and immunogenicity of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* vaccines prepared using different inactivators

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Author contributions

YW completed the inactivation of mycoplasma and prepared the manuscript. Immunogenicity evaluation of inactivated mycoplasma in mice was carried out by JW and TY. LW and BL helped with mycoplasma culture and the CCU assay. YY, ZF and GS modified the manuscript. QX and TK supervised and guided this work. All authors read and approved the final manuscript.

Student: [redacted] Date: 08 February 2023

Supervisor: [redacted] Date: 08 February 2023

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Abstract

Mycoplasmal pneumonia of swine (MPS) caused by *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is a chronic respiratory infectious disease of swine and has a significant impact on the economy. The main clinical symptom of the disease is cough, accompanied by reduced growth performance. Inactivated vaccine is used extensively to control *M. hyopneumoniae* infection worldwide. This study focused on a quantitative detection method and inactivation kinetics of *M. hyopneumoniae* in the process of vaccine production. In this study, an indirect competitive enzyme linked immunosorbent assay (ic-ELISA) was established and optimized using elongation factor thermo unstable (EF-Tu) as the target protein. EF-Tu protein is abundant on the surface of *M. hyopneumoniae*. The ic-ELISA assay was used to quantify the *M. hyopneumoniae* cultures at different growth stages, and compared with the traditional color changing unit (CCU) assay. The ic-ELISA assay results obtained for a growth curve were similar to that of the CCU assay indicating a strong correlation in the log phase. Also, a linear regression equation was established between the ic-ELISA and CCU assays in the log phase. The ic- ELISA method was used to evaluate the relative potency values of different batches of cultures over the internal reference vaccine to determine whether the cultures meet the antigen amount requirements for vaccine preparation. Compared with the CCU assay, the established ic-ELISA assay can quantitatively detect the antigen content in the process of vaccine production more quickly. Furthermore, this study also systematically compared the inactivation effects of, formaldehyde, thimerosal, β-propiolactone (BPL) and binary ethylenimine (BEI) on *M. hyopneumoniae*. Complete inactivation were achieved by 0.01% formaldehyde for 24 h at 37°C, 0.0008% thimerosal for 12 h at 37°C, or 0.02% BPL for 24 h at 4°C or 0.004% BEI for 24 h at 37°C. The immunogenicity of the antigens after inactivation was detected by mice immune test. The IgG antibody level of the mice immunized with the vaccine inactivated by 0.01% formaldehyde for 24 hours was significantly higher than those of the mice immunized with the vaccines inactivated by other solutions. The data indicated that formaldehyde can be used to inactivate *M.*

hyopneumoniae before vaccine preparation. In a conclusion, this study provides important reference value for the antigen quantitative detection and inactivation of *M. hyopneumoniae* for vaccine production.

Acknowledgements

After graduating from Nanjing Agricultural University, I worked in Jiangsu Academy of Agricultural Sciences for 10 years. Thanks to my teachers Guoqing Shao, Zhixin Feng and Qiyan Xiong for creating opportunities for me, as well as my supervisor Thandeka Khoza and KwaZulu Natal University for giving me the opportunity to further my studies. As time flies, my postgraduate career is coming to an end. I sincerely thank my supervisor Dr. Thandeka Khoza and Dr. Xiong Qiyan for the painstaking training during my post graduate studies.

I would especially like to thank my supervisor Dr. Thandeka Khoza. I was lucky that JAAS hosted her for three months and this provided me an opportunity work with her laboratory for that duration. Her rigorous working attitude and approachable manner made it easy for me to learn from her and ask for help, which improved my experimental design and english writing skills.

This thesis was completed under the careful guidance of my co-supervisor Dr. Xiong. I encountered many difficulties throughout the course of this study, however Dr. Xiong put forward valuable suggestions timely and improved the implementation scheme, so that I could complete the experiments successfully. Dr. Xiong's profound professional knowledge, excellent work style and approachable personality charm have a far-reaching impact on me. Therefore, I would like to express my deep gratitude and blessing to Dr. Xiong!

Very special thanks go to my teachers Guoqing Shao and Zhixin Feng for their guidance during my studies. I am also very grateful to Dr. Yanfei Yu for her help with experiment work and assisting in drafting of the dissertation. I am also appreciative of my colleagues (Jia Wang, Fei Hao, Li Wang, Beibei Liu, Ting Yuan, etc.) for their care and help!

Finally, I would like to thank my family for their meticulous care, understanding and support in my post graduate studies!

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Abbreviations

BPL	β-propiolactone
BEI	binary ethylenimine
BALF	broncho alveolar lavage fluid
BSA	bovine serum albumin
CCU	color changing unit
CFU	colony forming unit
CV	coefficient of variation
DNA	deoxyribonucleic acid
DMEM	dulbecco's modification of eagle's medium
EP	enzootic pneumonia
ELISA	enzyme-linked immunosorbent assay
EF-Tu	elongation factor thermo unstable
HRP	horseradish peroxidase
IFA	immunofluorescence assay
IHC	immunohistochemistry
ISH	in situ hybridization
Ig	Immunoglobulin
IPTG	isopropyl-β-d-thiogalactopyranoside
INA	iodonaphthyl azide
ic-ELISA	indirect competitive enzyme linked immunosorbent assay
MPS	mycoplasmal pneumonia of swine
MLST	multi-locus sequencing typing

MLV	modified live virus
MLVA	multiple-locus variable number tandem repeats analysis NET
MFI	mean fluorescence intensity
OD	optical density
PCR	polymerase chain reaction
PCV-2	porcine circovirus type 2
PBS	phosphate-buffered saline
PBST	Phosphate-buffered solution
pH	power of hydrogen
PRDC	porcine respiratory disease complex
PRRSV	porcine reproductive and respiratory syndrome virus
qPCR	quantitative PCR
RP	relative potency
RNA	ribonucleic acid
SPF	specific-pathogen-free
TMB	3,3′,5,5′ Tetramethylbenzidine
VNTRs	variable number of tandem repeats
WHO	World Health Organization

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Mycoplasma species are placed in the phylum Tenericutes, which are members of the class Mollicutes. They belong to the order Mycoplasmatales and the family Mycoplasmataceae (Nowak J., 1929). Mycoplasma species are the genus of bacteria, which are the smallest self-replicating organisms lacking a cell wall (Neimark H.C., 1986). Due to regressive evolution, this bacterium requires cholesterol for growth and has a small genome with low G+C content (23%-40%). The UGA codon is assigned for tryptophan in Mycoplasma species (Weisburg *et al*., 1989). UGA is a nonsense or termination (opal) codon throughout prokaryotes and eukaryotes. Yamao *et al.* (1985) reported *Mycoplasma capricolum* having a genome only 20-25% the size of the Escherichia coli genome. The small genome results in their inability to rapidly synthesize ribosomes and high dependence on nutrients from the host (Yamao *et al*.,1985). Therefore, mycoplasmas usually have a slow growth rate (Pieters, 2019).

Six species of Mycoplasma in the Mollicutes class are commonly found in pigs. These are *Mycoplasma hyopneumoniae, Mycoplasma hyorhinis, Mycoplasma flocculare, Mycoplasma hyosynoviae, Mycoplasma hyopharyngis* and *Mycoplasma suis*. *M. hyopneumonia* causes enzootic pneumonia (EP) in pigs and has been reported worldwide (Pieters and Maes, 2019). It can cause a decline in production performance, high health care costs and differences in growth rates, thus resulting in huge economic losses (Maes *et al*., 2021b). Furthermore, this pathogen is a primary pathogen of the porcine respiratory disease complex (PRDC), in which it interacts with other pathogens, such as *Pasteurella multocida, Actinobacillus pleuropneumoniae,* porcine reproductive and respiratory syndrome virus, swine influenza virus and porcine circovirus type 2 (Gulliksen *et al.*, 2021). Both EP and PRDC cause great economic losses in pig farms worldwide.

M. hyorhinis is another important Mycoplasma pathogen in swine. It is ubiquitous in pig herds, especially in nursing pigs, and commonly inhabits the upper respiratory tract and tonsils of pigs. Although most infections are subclinical, in some cases *M. hyorhinis* can induce polyserositis, polyarthritis and other inflammations (Martinson *et al.*, 2018a; Morita *et al.*, 1999). *M. flocculare* was described by Meyling and Neils in 1972 as a new species based

on growth inhibition and metabolic inhibition tests (Meyling and Neils, 1972). It closely resembles *M. hyopneumoniae* in characteristics and appearance, with no central core to the colony. However, *M. flocculare* strains are usually grow slower than *M. hyopneumoniae*. *M. flocculare* is found in the nasopharynx of the pig and appears to be nonpathogenic (Friis, 1973; Fourour *et al.*, 2019). *M. hyosynovia*, first described by Richard Ross and Judith Karmon in 1970, was found in respiratory tracts and in the joints of pigs with arthritis. (Ross and Duncan, 1970). The joint disease associated with infection is mostly seen in pigs older than 10 weeks (3–5 months) and can be acute, with swelling particularly visible in the hock joints, difficulty moving, stiffness, lameness and an arched back (Friis *et al.*, 1992; Nielsen *et al.*, 2001). *M. hyopharyngis* was first described by Eriksen *et al.* in 1986 (Erickson *et al.*, 1986). Kobisch and Friis noted *M. hyopharyngis* to be an arginine-metabolizing mycoplasma from nasal and pharyngeal samples from pigs (Kobisch and Friis, 1996). *M. suis* (formally *M. hemosuis*), which was recognized in the Midwest of the United States of America (USA) in 1931 (Kinsley, 1932; Kinsley and Ray, 1934), is now recognized as the name for the agent of porcine eperythrozoonosis or porcine hemoplasmosis (Neimark *et al.*, 2001). This is seen worldwide in domestic and wild pigs and is considered an economically significant infection in swine production (Hoelzle *et al.*, 2014). The following content mainly introduces the etiology, epidemiology, clinical symptoms, diagnosis, vaccine and key technologies in vaccine production of *M. hyopneumoniae*.

1.2 Etiology of *M. hyopneumoniae*

In 1965, Maré and Switzer isolated a microorganism from EP cases in the United States that can be cultured in the improved broth described by Goodwin and Whittlestone (Mare and Switzer, 1965). Furthermore, they were able to induce lesions of EP with the microorganism. Maré and Switzer cultured the microorganism from broth cultures and lung suspensions of swine suffering from pneumonia. They obtained colonies on solid medium containing 1% agar, these colonies looked different to *M. hyorhinis* and were named *M. hyopneumoniae*.

Traditionally, isolation is considered the "gold standard" method to prove the existence of *M. hyopneumoniae* in lung tissue. However, due to the specific nutritional needs and harsh culture conditions, culturing of this microorganism is time-consuming and laborious, taking as long as 10–15 days to become visible. This is exacerbated by an overgrowth of contaminating bacteria such as *M. hyorhinis* which grows quickly and overwhelms the slow-growing *M. hyopneumoniae*. The difficulty experienced in culturing *M. hyopneumoniae*

limits our understanding of the toxicity of *M. hyopneumoniae* and the pathogenesis of EP.

In 1975, Niels Friis developed the most commonly used liquid broth medium for culturing mycoplasma isolating *M. hyopneumoniae* from clinical samples （Friis, 1975）. This liquid broth medium is known as Friis medium, which contains fresh yeast extract, brain heart broth, thallium acetate, phenol red (as a pH indicator) and swine or horse serum (Armstrong, 1994). Generally, it is very difficult for *M. hyopneumoniae* to grow on solid agar medium. It usually takes several weeks of passage through liquid medium for several generations before it can form colonies on solid agar medium. Restricted growth on solid medium is related to agar purity and its ability to inhibit growth or retain some nutrients (Cook *et al.*, 2016). To prevent contamination by *M. hyorhinis* when culturing *M. hyopneumoniae*, Friis medium is supplemented with 5% anti-*M. hyorhinis* serum and 500 μg/ml cycloserine (Kobisch, 1996). Alternatively, kanamycin may be added to selective medium for *M. hyopneumoniae* to inhibit the growth of *M. hyorhinis*. (Cook *et al.*, 2016).

The ideal samples for the isolation of *M. hyopneumoniae* are lung tissues with typical pneumonic lesions and the surrounding normal tissues from swine in the acute infection stage (Armstrong, 1994), as well as bronchial swabs or bronchoalveolar lavage fluid (BALF). The viable cell count of *M. hyopneumoniae* in chronic cases is low. To avoid contamination by other bacteria, it is recommended to filter the suspension through a 0.45 μM filter. This filtered suspension can be diluted 10 times with liquid medium and cultured at 37 ℃ for days to weeks. The culture time is usually determined by the color change of the phenol red indicator. When the liquid medium turns yellow and there is no turbidity, the culture can be harvested and passaged or identified. Subsequently, color changing unit (CCU) is the most commonly used to quantify growth of measurement method as *M. hyopneumoniae* grows slowly. However, method is indirect, subjective and low in accuracy (Calus *et al.*, 2010). Studies have reported that ATP photometry, flow cytometry and/or qPCR detection method can provide support for CCU assays (Assuncao *et al.*, 2005; Calus *et al.*, 2010; Garcia-Morante *et al.*, 2018).

1.3 Epidemiology

It was been reported that more than 70% of pigs are infected with *M. hyopneumoniae*

worldwide (Pieters, 2019). Interestingly, most countries do not have direct detection data on the existence of EP infection in pigs, except that the mycoplasma elimination project in Denmark has carried out serological monitoring on approximately 2500 pig herds. Most infection related data came from the evaluation of the effect of vaccines and antibiotics. In some countries, the common practice of detecting *M. hyopneumoniae* was to assess lung lesions in slaughterhouses. Nevertheless, there is a lack of pathognomonic lesions and the potential for lesions to heal prior to market age (Noyes, 1990), so it was difficult to accurately reflect the prevalence of pathogens in this area by the evaluation of lung lesions in slaughterhouses. It is encouraging that some countries such as Switzerland (Stark *et al.*, 2007), Norway (Gulliksen, 2019) and Finland (Rautiainen *et al.*, 2001) have announced the successful elimination of porcine *M. hyopneumoniae*, but the treat remains since this pathogen has been found in wild boars in Switzerland (Kuhnert *et al.*, 2011; Kuhnert, 2014).

The incidence rate of pigs infected with *M. hyopneumoniae* may be relatively low, but the infection rate could very high. Once EP infection occurs in pigs, the infection would persist for a long time. The transmission efficiency (RN) of *M. hyopneumoniae* is low (Meyns, 2004; Meyns *et al.*, 2011), therefore the pathogen does not infect all pigs in the pig herd immediately. A small number of pigs are infected first, and a considerable number of uninfected pigs could be infected upon contact with pigs that were infected. Recent studies have indicated that approximately 20% of gilts are still negative for *M. hyopneumoniae* up to the first farrowing, regardless of being introduced into endemically infected farms (Michiels *et al.*, 2018).

Molecular epidemiology studies can be used to distinguish between different *M. hyopneumoniae* strains. A single strain of *M. hyopneumoniae* has always been demonstrated in areas with a low prevalence of *M. hyopneumoniae*-positive herds (Mayor *et al.*, 2007; Vranckx *et al.*, 2012). In contrast, in high-density areas, various strains have been frequently found in individual swine herds (Michiels *et al.*, 2017; Nathues *et al.*, 2011). Another study suggested that the pigs in the herds containing multiple strains of *M. hyopneumoniae* showed severe lesions in their lungs at slaughter (Michiels *et al.*, 2017).

1.4 Clinical signs and lesions

M. hyopneumoniae is a respiratory disease which is distributed worldwide and results in a substantial economic impact. Primarily, symptoms of the disease include coughing and

negatively impacts growth. Lesions caused by the pathogen are primarily found in the apical, cardiac and accessory lobes. Lesions in the lungs can be detected 7–10 days after inoculation (Kobisch *et al.*, 1993) and gradually worsen during 2–4 weeks post-infection. The lesions are fawn/pink or plum colored, which can be distinguished from normal tissues (Rodriguez *et al.*, 2004). The most obvious clinical sign is a dry cough in nursery, grower, and/or finisher pigs. Studies show that the dry cough presents itself as a symptom 1–2 weeks after infection with a high dose of *M. hyopneumoniae* (10^7 - 10^8 CCU/pig) (Marois *et al.*, 2007; Woolley *et al.*, 2012). Dry coughs may continue for several weeks, reaching its maximum at 4-5 weeks after infection (Garcia-Morante *et al.*, 2016b; Kobisch *et al.*, 1993). However, sole infection by *M. hyopneumoniae* is rarely observed under commercial conditions since the participation of other contaminating pathogens is very frequent and leads to increased severe clinical signs such as severe respiratory distress, fever, prostration, reduced appetite, and eventually mortality (Maes *et al.*, 2018; Opriessnig *et al.*, 2011).

1.5 Diagnosis

The diagnosis of *M. hyopneumoniae* infection in vivo is very difficult, mainly because the infection site is invisible, and the pathogen isolation may be challenging. The initial symptom of respiratory disease caused by this pathogen is that a certain number of growing pigs or finishing pigs show intermittent dry cough of different severity. During autopsy or slaughter examination, *M. hyopneumoniae* causes visible red to purple consolidation areas in the lung. These areas are usually distributed on both sides of the apical lobe, heart lobe, middle lobe and top of the diaphragmatic lobe (Garcia-Morante *et al.*, 2016a).

Common methods to detect *M. hyopneumoniae* in tissues include the immunofluorescence assay (IFA), immunohistochemistry (IHC) and in situ hybridization (ISH) (Doster and Boh, 1988; Kwon *et al.*, 2002; Opriessnig *et al.*, 2004; Sarradell *et al.*, 2003). IFA and IHC are both methods to detect specific target proteins, while ISH is used to detect target DNA. The colonization degree and tissue localization of *M. hyopneumoniae* can be determined by detecting formalin fixed samples by IHC and ISH. IFA is usually used to determine the tissue localization of *M. hyopneumoniae* in frozen tissue samples. The advantages of using PCR include high accuracy, fast turnaround time and high throughput.Various PCR methods including regular PCR, nested PCR, and real-time PCR have been developed and validated in clinical practice (Dubosson *et al.*, 2004; Strait *et al.*, 2008). However, the selection of *M. hyopneumoniae* samples for PCR detection is

challenging. Lung tissues and the bronchi exhibit gross lesions and are the most sensitive samples. Other clinical samples usually include oral fluids, swabs collected from nasal cavity, tonsil, or tracheobronchial, and tracheobronchial lavages (Calsamiglia *et al.*, 1999; Fablet *et al.*, 2010; Pieters *et al.*, 2017; Sibila *et al.*, 2004).

The target genes of *M. hyopneumoniae* detected by PCR mainly include the genes coding 16S rRNA, P36, P46, and P97. Mattsson designed primers targeting the 16S rRNA gene and established a PCR detection method for *M. hyopneumoniae*. This method can detect nasal swabs with *M. hyopneumoniae* under natural infection conditions in pigs (Mattsson *et al.*, 1995). Since then, this method has been widely used by researchers from different areas in different countries such as the United States, Spain, Canada, Germany, France and Denmark. The 16s rRNA primers has been used as an outer primer to design a nested PCR detection method and the results demonstrated that nested PCR is a valuable adjunct in the diagnosis of *M. hyopneumonia*e infection (Calsamiglia *et al.*, 2000).

The P36 protein, also known as lactate dehydrogenase, is a specific immune dominant protein on *M. hyopneumoniae* and this gene is highly conserved in the evolution of *M. hyopneumoniae*. Subsequently, PCR of the p36 gene has also been widely used in the detection of *M. hyopneumoniae* and has a positive detection rate of 100% for *M. hyopneumoniae* in affected lung tissues and with no false-positive result in 62 normal lung tissues (Caron *et al.*, 2000). Other genes that has been used for specific determination of *M. hyopneumoniae* include P46, P97 and P102 which use various PCR techniques (Table 1.1).

Table1. 1 Summary of PCR detection technology of *M. hyopneumoniae*

Reference	The type of PCR	Amplification		The minimum detectable concentration	Clinical samples
		Gene	Length（bp）		
(Harasawa *et al.*, 1991)	S	Repeat unknown sequence	520	5 ng or 1000 CFU/ml	/
(Artiushin *et al.*, 1993)	S	Unique hypothetical sequence	456	1-10 pg DNA	Alveolar lavage fluid, lung tissue
(Mattsson *et al.*, 1995)	S	16S rRNA	200	1000 copy	/
(Stemke *et al.*, 1994)	S	16S rRNA	649	5 CFU	Nasal swab
(Blanchard *et al.*, 1996)	S	Hypothetical ABC transporter	1561	500 fg	Tracheobronchial lavage fluid
(Stark *et al.*, 1998)	N	MHYP1-03-950 repeat sequence	808	1-cell filter membrane	Filtered air sample
(Baumeister *et al.*, 1998)	S	/	853	100 CFU/ml	Alveolar lavage fluid
(Calsamiglia *et al.*, 1999)	N	16S rRNA	352	80 cells	Nasal swab
(Verdin *et al.*, 2000)	N	Hypothetical ABC transporter	706	1 fg	Tracheobronchial lavage fluid, nasal swab
(Caron *et al.*, 2000)	S	Intergenic sequence P36	948	50 pg	Lung tissue, tracheobronchial lavage fluid
	S	Intergenic sequence P46	580	0.5 ng	Nasal swab
	M	P36 and P46	948/580	/	/
(Kurth *et al.*, 2002)	N	Unique hypothetical sequence	240	0.5-1 fg	Bronchial brush, alveolar lavage fluid
(Dubosson *et al.*, 2004)	RT	MHYP1-03-950 repeat sequence	808	1 fg	Bronchial swab
		Hypothetical ABC transporter	706	1 fg	/
(Stakenborg *et al.*, 2006)	M	16S rRNA	1000	1 fg	/

(Hugh et al., 2007)	S	16S rRNA	649	0.18 CFU/g	Lung tissue
(Strait *et al.*, 2008)	RT	P165 and P183	529/90	2.5 fg	*M. hyopneumoniae* isolates
(Fourour *et al.*, 2018)	RT	P102	150	14 genome equivalents μl^{-1}	Lung tissue

S: simple PCR; N: nested PCR; M: multiplex PCR; RT: real-time fluorescence quantitative PCR.

The serological method is an effective tool to determine the incidence rate of *M. hyopneumoniae* in pigs. ELISA technology is the most commonly used method to detect *M. hyopneumoniae* infection. In general, various ELISA assays are commercially available for the detection of *M. hyopneumoniae*-specific IgG in serum samples. The assays differ in the types of ELISA (i.e., direct, indirect, or blocking) and the types of antigen used (i.e., whole cell or individual proteins). IgG antibody responses are usually evaluated in pig serum, which can be detected as early as 21 days after infection (Pieters *et al.*, 2017). Other types of antibodies used to detect *M. hyopneumoniae* exposure include IgM and IgA (Neto *et al.*, 2014; Pieters *et al.*, 2017). Researchers have evaluated IgA antibody responses with different samples, including nasal swab, pen-based oral fluid, and tracheobronchialar or bronchoalveolar lavage fluid samples *(Bai et al., 2018; Bjustrom-Kraft et al., 2018; Garcia-Morante et al., 2017; Pieters et al*., 2017). Thus far, it is impossible to distinguish the antibody response caused by natural infection with *M. hyopneumoniae* from that caused by a commercial vaccine. Fortunately, it has recently been found that IgA detection can distinguish infected pigs from immunized pigs without obvious interference of passive immunity (Bai *et al.*, 2018). For these reasons, serology is a useful diagnostic method mainly in populations and when vaccination status is known.

1.6 Vaccines against *M. hyopneumoniae*

At present, the methods to control EP include antibiotic treatment and vaccination. Since *M. hyopneumoniae* colonizes respiratory epithelial cells and does not enter the blood, drug treatment is limited to prevent and control the disease. Therefore, vaccination has been used to control EP worldwide. Vaccination can improve the health of pigs and reduce the use of antibiotics (Tao *et al.*, 2019). At present, registered vaccines against *M. hyopneumoniae* include inactivated vaccines and attenuated vaccines (Table 1.2)

Table1. 2 Approved MPS vaccines.

Common name	Strain of Mhp	Manufacturer	Approval year
MPS inactivated vaccine	J	Boehringer Ingelheim Vetmedica, GmbH	2017
	J	Boehringer Ingelheim Vetmedica, Inc.	2017
	P	Protatek International, Inc.	2016
	J	Intervet International, B.V.	2016
	DJ-166	China Animal Husbandry Industry Co., Ltd.	2016
	BQ14	Merial, Inc.	2015
	J	Laborations HIPRA, S.A.	2015
	P-5722-3, I	Zoetis, Inc.	2014
	P-5722-3	Harbin Pharmaceutical Group Holding Co., Ltd.	2014
	P-5722-3, II	Zoetis, Inc.	2013
	J	Boehringer Ingelheim Vetmedica, GmbH	2013
	P	Protatek International, Inc.	2012
	J	Boehringer Ingelheim Vetmedica GmbH	2012
	J	Intervet International, B.V.	2011
	BQ14	Merial, Inc.	2011
	J	Laborations HIPRA, S.A.	2010
	P-5722-3	Pfizer, Inc.	2010
MPS compound adjuvant inactivated vaccine	P	Protatek International, Inc.	2017
	P	Protatek International, Inc.	2012
MPS live vaccine	RM48	EBVAC	2017
	168	Nanjing Tianbang Bioindustry Co., Ltd.	2016
	168	Fuzhou Da Bei Nong Biotech	2016
	168	Guizhou Fu Si Te Biotech	2016
	168	QYH Biotech Company, Ltd.	2015
	RM48	QiLu Animal Health Products Co., Ltd.	2015
	RM48	Shandong Lvdu Biotechnique Industry	2015

1.6.1 Inactivated vaccines

Inactivated vaccines are commonly used to control mycoplasmal pneumonia of swine (MPS). Since the MPS inactivated vaccine was registered in the United States in the 1990s, a number of studies have confirmed that the vaccine could bring great economic benefits to the pig industry. In the United States, more than 85% of pigs are vaccinated to control the disease.

Commercial inactivated vaccines are mainly composed of adjuvants and inactivated whole bacteria (Maes *et al*., 2018). Strain J is the standard reference strain of *M. hyopneumoniae*, so most vaccines are based on strain J. The strain was a highly virulent strain isolated from sows with mild endemic pneumonia in 1963 (Goodwin and Whittlestone, 1963) however, the strain lost its virulence gradually after continuous passages in vitro (Zielinski and Ross, 1993). The strains used to prepare inactivated vaccines against *M. hyopneumoniae* include strain J, strain P, strain BQ14, strain P - 5722 - 3 (Tao *et al*., 2019). In China, domestic inactivated vaccines against *M. hyopneumoniae* have been developed in recent years. These vaccines, including strain DJ - 166, strain HN0613, strain NJ, strain CJ and strain SY, have a high market share in China (Mu Guanghui, 2021).

1.6.2 Live vaccines

Live attenuated vaccines can produce efficient local and systemic immune responses and have been approved for use in Mexico and China (Feng *et al*., 2013). The strain used for the live attenuated vaccines in Mexico is the temperature sensitive mutant of *M. hyopneumoniae* (LKR strain) (VaxSafe) ® MHYO, Avimex), which is nasally administered once in 3-day-old pigs. The attenuated vaccine strain in China was strain 168, which was isolated in 1974. The strain was isolated from the Erhualian pig (a local pig species in China that is very sensitive to *M. hyopneumoniae*) (Feng *et al*., 2010). The virulence of the strain was weakened gradually by continuous alternative passages in vivo and in vitro with pigs and KM2 cell-free medium (improved Friis medium) respectively. After 300 passages, the strain was completely weakened, and its antigenicity was retained. Whole-genome sequencing showed that the attenuated strain contained 60 insertion sites and 43 deletion sites. Mutations in genes related to metabolism and growth may contribute to the attenuated virulence in the strain 168, in addition to variations previously described in *M. hyopneumoniae* adhesins (P97, P102, P146, P159, P216, and LppT), cell envelope proteins (P95), cell surface antigens (P36), and secreted proteins and chaperone protein (DnaK) (Liu *et al*., 2013). The vaccine has high antigenicity and can produce local and efficient mucosal immunity through intrapulmonary

injection (Feng *et al.*, 2010). The protective efficacy of the attenuated vaccines was higher than 80% (Feng *et al.*, 2010).

1.6.3 Combined inactivated vaccines

Clinically, *M. hyopneumoniae* is mostly found in coinfection with other pathogens such as PCV2, porcine reproductive and respiratory syndrome (PRRSV) and *Haemophilus parasuis*(Zhu *et al*., 2021). Infection with PRRSV aggravated lung lesions caused by *M. hyopneumoniae* (Escobar *et al*., 2004). Combination vaccine prepared against PRRSV and *M. hyopneumoniae* can effectively control a double infection with these two pathogens *(Bourry et al*., 2015; Stricker *et al.*, 2013). PCV2 and *M. hyopneumoniae* combined vaccine development have showed good results in the prevention of both these diseases and have thus realized commercialization (Duivon *et al.*, 2018; Jeong *et al.*, 2016; Pujols *et al.*, 2016). Other combined vaccines for *M. hyopneumoniae* with other pathogens include *M. hyopneumoniae* and *Actinobacillus pleuropneumoniae* (Wongnarkpet *et al.*, 1999) as well as *M. hyopneumoniae* with *Bordetella bronchiseptica* (Yim *et al*., 2017). It was observed that *M. hyopneumoniae-Bordetella bronchiseptica* combined vaccine enhanced the production of *M. hyopneumoniae* antigen-specific IgG in mice (Yim *et al*., 2017).

1.6.4 Genetically engineered vaccines

In the early stages of the development of genetically engineered vaccines for *M. hyopneumoniae*, most researchers use one antigen that are assumed to be critical in the process of pathogenic infection. The proteins of these antigens are mainly P97, P102, P95, P46, P65, NRDF, P42, P159. The selection of these proteins is mainly based on the infection mechanism of *M. hyopneumoniae*, which interacts with the cilia of host respiratory epithelial cells (Marchioro *et al.* ,2014; de Oliveira *et al.*,2017).

The monovalent vaccine or bivalent vaccine of *M. hyopneumoniae* provides limited protective effects. Therefore, studies of multivalent vaccines have become a hot spot in recent years. Chen *et al*. used DNA and protein combination vaccines containing five antigens (NRDF, P97, P97R1, P36 and P46) into mice through intramuscular and subcutaneous injection, which produce significant humoral immunity and a Th1-type cellular immune response (Chen *et al.*, 2006). Ma *et al.* intramuscularly injected the *Salmonella cholerae* vaccine containing five adhesins (P36, P46, P65 P97R1, and NRDF) of *M. hyopneumoniae* into mice, and the results showed a relatively good immune response (Ma *et al.*, 2011). De

Oliveira *et al.* constructed a recombinant multivalent chimeric vaccine containing four antigens (P71R1, P46, P95 and P42) of *M. hyopneumoniae*, and achieved satisfactory results in the evaluation of immune effects on mice (de Oliveira *et al*., 2017).

1.7 Key technologies in vaccine production

1.7.1 Quantitative detection method

Quantifying the culture antigen of *M. hyopneumoniae* in the shortest possible time is very important in *M. hyopneumoniae* vaccine preparation. Currently, CCU and colony forming unit (CFU) assays are commonly used for mycoplasmas quantification (Garcia-Morante *et al.*, 2018). Although the CFU detection is used widely for bacterial quantification, but it is not suitable for a variety of mycoplasma species because they are difficult to be cultured in solid media. Therefore, the alternative non-agar based method, the CCU assay, is most commonly used for *M. hyopneumoniae* quantification (Garcia-Morante *et al*., 2018). However, this method is time-consuming, taking two weeks to determine the results, and thus does not meet the time requirements of the vaccine manufacturers. In recent years, several methods, including ATP luminometry (Calus *et al*., 2010), flow cytometry assay (Assuncao *et al*., 2005), quantitative real-time PCR (qPCR) (Sibila M, 2012), fluorescent double-stranded DNA (dsDNA) staining (Garcia-Morante *et al*., 2018), and enzyme-linked immunosorbent assays (ELISAs) (Tian, 2017), have been used to quantify *M. hyopneumoniae*. These methods have their own characteristics and certain scopes of application. Using ELISA methods to quantify the antigen is a suitable choice in inactivated vaccine antigen determination and has already been used for different kinds of vaccines (Chabaud-Riou *et al*., 2017; Gairola *et al*., 2020; Sharma *et al.*, 2019).

1.7.2 Antigen inactivation method

Another important procedure in the production of inactivated vaccines is the inactivation of pathogens. Inactivation in the sense of biological products refers to the process of using physical or chemical methods to destroy the biological activity of microorganisms and the reproductive and pathogenic ability of pathogenic microorganisms, which still retain reactivity and immunogenicity (Nicholas *et al.*, 2009). There are many factors affecting the complete inactivation of antigens. These factors include; (1) Types and characteristics of microorganisms; different kinds of microorganisms, such as bacteria, viruses, fungi, and even gram-negative and gram-positive bacteria, have completely different sensitivities to various

inactivating solutions (Turner *et al.*, 1970). (2) Inactivation temperature; the inactivation rate of the same concentration of inactivator is directly proportional to the temperature (David *et al*., 2010). When the temperature is higher, the inactivation process is faster. (3) Concentration of inactivating solution; generally, the concentration of the inactivator should increase as the concentration of antigen increases (David *et al.*, 2010). (4) Inactivation time and inactivation temperature are closely related to the concentration of the inactivator (Loveday *et al.*, 2021). Generally, with the increasing of inactivator concentration and action temperature, the time required for inactivation is shortened. However, to ensure quality, low concentrations, low temperatures and short treatment times are generally preferred (Loveday *et al.*, 2021). (5) The inactivation effect enhanced by adjusting the pH of the solution before thermal denaturation to 6.5 or higher (Takahashi *et al.*, 2020).

At present, the commonly reported antigen inactivation methods for mycoplasma vaccine include formaldehyde, thiomersal, β-propiolactone (BPL), binary ethylenimine (BEI) (David *et al.*, 2010; Martinson *et al.*, 2019). The efficiency of inactivation with beta-propiolactone, formalin, cetyltrimethylammonium bromide, Triton X-100, and sodium deoxycholate was evaluated on 22 mycoplasma species. 0.5% Triton X-100 or 0.5% sodium deoxycholate demonstrated the most efficient inactivation in the mycoplasmas (David *et al.*, 2010). Cetyltrimethylammonium bromide could also inactivate all mycoplasmas rapidly (in less than 30 min) at concentrations above 0.08%. (David *et al.*, 2010). Tamiya *et al.* reported that *M. pneumoniae* could be inactivated with 0.16% formaldehyde (Tamiya *et al.*, 2020). Gong *et al.* evaluated protection efficacy of an inactivated *M. synoviae* vaccine with different adjuvants in broilers against a Chinese field isolate (CHN-BZJ2-2015) and the *M. synoviae* cultures were inactivated with final 0.1 M BEI for 24 h at 37 °C (Gong *et al.*, 2020b). Mwirigi *et al.* reported that *Mycoplasma mycoides subsp. mycoides* culture was inactivated by adding 0.7% (v/v) formaldehyde with incubation overnight at 37 °C *(Mwirigi et al.*, 2016a). Wei *et al.* reported that the vaccine preparation contained 1 $\times 10^{10}$ CCU/mL *M. hyorhini* and $10^{5.5}$ TCID50/mL PCV2, and the two pathogens were inactivated with a concentration of 2% formaldehyde at 24 h in a 37 °C humidified incubator (Wei *et al.*, 2020). Also,*M. hyorhinis* was inactivated with binary ethylenimine (Martinson *et al*., 2018b). The inactivation method determines the degree of damage and immunogenicity of antigens, so it is particularly important for vaccine production.

1.8 Objective of the study

Inactivated vaccines are very important for the prevention and control of mycoplasma diseases (Tao *et al.*, 2019). The quantitative detection and inactivation of *M. hyopneumoniae* are both of important steps in inactivated vaccine development. For the titer detection method of *M. hyopneumoniae*, the CCU assay is the most commonly used method at present, but which is time-consuming and laborious. There are some reports about different detection methods, such as ATP, CCU, CFU, and quantitative real-time PCR (qPCR) (Garcia-Morante *et al.*, 2018). However, these methods are not suitable for the vaccines. For the inactivation of *M. hyopneumoniae*, the commonly used inactivators were lack of systematic evaluation methods. Aiming at these two key points of vaccine production, the purpose of this study was to develop a rapid method for detecting the antigen of *M. hyopneumoniae* and screen the best inactivator to provide the reference evidences for vaccine production.

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CHAPTER 2. DEVELOPMENT A RAPID METHOD FOR QUANTITATIVE DETECTION OF MYCOPLASMA HYOPNEUMONIAE ANTIGEN

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2.1 Abstract

Inactivated *Mycoplasma hyopneumoniae* vaccine is used extensively to control *M. hyopneumoniae* infection worldwide. Quantification techniques are essential in the process of standardizing and validating vaccines. In this study, we developed and optimized an indirect competitive enzyme linked immunosorbent assay (ic-ELISA) for the rapid quantification of *M. hyopneumoniae* antigen during vaccine production. Briefly, whole *M. hyopneumoniae* antigen was coated onto microtiter plates, and a polyclonal antibody against *M. hyopneumoniae* recombinant elongation factor thermo unstable (EF-Tu) protein was prepared and added with the samples to be tested. The methods were optimized and showed significant reproducibility, with coefficients of variation of 4.01% and 6.14% for the intra-and inter-assays, respectively. Quantification of *M. hyopneumoniae* cultures at different growth stages using the ic-ELISA test showed a similar curve to that of the traditional color changing units (CCU) assay, with a delay in the time when the amount reached the peak and started to fall. In the inactivated vaccine production process, the cultures could be harvested later than that for the live vaccine, at about 12 h after the end of the logarithmic growth phase. Different batches of cultures were measured for their relative potency value compared with the in-house reference vaccine, which was used to determine whether the cultures met the antigen amount requirements for vaccine preparation. The curves of the CCU titer and ic-ELISA titer in the logarithmic phase correlated strongly and a linear regression equation was established to calculate the CCU values rapidly using the ic-ELISA results. In conclusion, an ic-ELISA method was established to rapidly assess the amount of antigen in an *M. hyopneumoniae* culture during the vaccine production process.

2.2 Introduction

M. hyopneumoniae is a widespread respiratory pathogen in pigs, and is considered as the primary agent of porcine enzootic pneumonia (EP), a chronic disease in pig herds (Maes *et al.*, 2018; Holst *et al.*, 2015; Simionatto *et al.*, 2013). EP causes significant economic losses to the pig industry every year. Vaccines are used worldwide as one of the important measures to prevent and control EP (Maes *et al.*, 2018; Holst *et al.*, 2015; Simionatto *et al.*, 2013; Xiong *et al.*, 2014). Both live and inactivated vaccines are available commercially. Live vaccines are only approved by a few countries, including China and Mexico (Feng *et al.*, 2010), while inactivated vaccines are used commonly worldwide (Tao *et al.*, 2019). Vaccines can effectively reduce lung lesions, improve the feed conversion rate, and shorten the time of slaughter. In the large-scale production of *M. hyopneumoniae* for vaccine preparation, it is necessary to quantify the antigen in the culture in the shortest possible time. Currently, color changing units (CCU) and colony forming units (CFU) assays are used to quantify mycoplasmas (Garcia-Morante *et al.*, 2018). The CFU technique is used widely for bacterial quantification; however, it is not suitable for a variety of mycoplasma species or strains because they are difficult to culture on solid medium, or the colonies are too small for accurate counting. Therefore, the alternative non-agar based method, the CCU assay, is used as the gold standard for *M. hyopneumoniae* quantification (Garcia-Morante *et al.*, 2018). However, CCU assay is time-consuming, taking at least 14 days to determine the results. It does not meet the needs of vaccine manufacturers to shorten the production process or could result in failure to stop the production process in time in cases of poor quality vaccine products, thus causing unnecessary losses. Furthermore, CCU determination could be affected by various factors, such as serum, the container, and operations, resulting in obvious defects in accuracy and reproducibility (Wei *et al.*, 2012). In addition, CCU only detects live bacteria, and is not suitable for use when the bacteria have been inactivated. For inactivated vaccine antigen determination, the enzyme-linked immunosorbent assay (ELISA)-based protein quantification method is a suitable choice and has already been used for different kinds of vaccines (Gairola *et al.*, 2020; Sharma *et al.*, 2019; Martine *et al.*, 2017). However, very few studies on ELISA detection of *M. hyopneumoniae* antigens have been reported to date. In a previous study in our laboratory, a double-antibody sandwich ELISA targeting the P46 protein to determine the amount of the *M. hyopneumoniae* antigen in the inactivated vaccine was established (Tian *et al.*, 2017). However, our later research found that P46 was

not only expressed on the surface of *M. hyopneumoniae*, but also secreted into the supernatant. Moreover, the amount of P46 protein secreted into the supernatant was much higher than that expressed on the surface (unpublished data). High titer, usually around 10^8–10^9 CCU/mL, is required for the inactivated *M. hyopneumoniae* vaccine. Therefore, ultrafiltration concentration is often used in the production process. During ultrafiltration concentration, most of the medium supernatant will be discarded. Consequently, there is a significant difference between the change in the P46 content and the change in the amount of bacteria before and after concentration, which restricts the application of the P46-ELISA method. The identified adhesins, such as P36, P97, P159, P146, and P216 (Frey *et al.*, 1994; Zhang *et al.*, 1995; Burnett *et al.*, 2006; Mayor *et al.*, 2007; Wilton *et al.*, 2009), are important antigens of *M. hyopneumoniae*. However, because of their expression differences, not all the surface exposed proteins are suitable as detection targets. In our previous work, we identified that elongation factor thermo unstable (EF-Tu) acts as an important adhesin on the cell surface and interacts with different components of the host (Yu *et al.*, 2018; Yu *et al.*, 2020). The expression level of EF-Tu on the surface of the bacteria is significant (Yu *et al.*, 2018), and its expression in the supernatant is very low (Appendix, Sup. Table 1). This suggested the possibility of using EF-Tu as the target to develop a new ELISA to determine the concentration of *M. hyopneumoniae* antigen. In the present study, an indirect competitive ELISA (ic-ELISA) was established to rapidly quantify an *M. hyopneumoniae* antigen. The results of ic-ELISA were compared with those of the CCU assay at different growth stages, and the relationship between them analyzed.

2.3 Materials and methods

2.3.1 *M. hyopneumoniae* strain and medium for culturing

M. hyopneumoniae NJ, a strain used to produce an inactivated *M. hyopneumoniae* vaccine that is approved for marketing in China, was used to produce the cultures in this study. *M. hyopneumoniae* was cultured with KM2 medium (Liu *et al.*, 2013) containing 20% (*v*/v) swine serum. The culture was incubated at 37 °C until the color turned yellow, at which point *M. hyopneumoniae* was harvested by centrifugation at 15000 ×*g* for 20 min at 4 °C. The precipitate was resuspended in phosphate-buffered saline (PBS) and then frozen at -70 °C until used.

2.3.2 CCU assay

Previously described conditions were followed to assess the CCU value (Calus *et al.*, 2010). Briefly, ten-fold serial dilutions (to 10^{-11}) of the *M. hyopneumoniae* culture in KM2 medium were performed, and incubated at 37 °C statically for 2 weeks. The highest dilution at which color changes was regarded as the endpoint and the titer was determined as CCU/mL. The CCU titer was calculated from three repeated measurements.

2.3.3 Preparation of polyclonal antibodies

The gene encoding EF-Tu (*M. hyopneumoniae* 168_533) was cloned into the vector pET-32a using the homologous recombination technique (Yu *et al.*, 2018). The reconstructed plasmid was transformed into *Escherichia coli* BL21 (DE3) for isopropyl-β-d-thiogalactopyranoside (IPTG)-induced expression. The induced recombinant protein was purified using Ni-chelating chromatography (GE Healthcare, Chicago, IL, USA). Polyclonal antibodies against the recombinant EF-Tu were then prepared according to a previously published protocol (Yu *et al.*, 2018). A one-month-old New Zealand white rabbit was immunized a total of three times with 1 mg of purified recombinant protein emulsified in Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) at 2-week intervals. Sera were collected 1 week after the third immunization. The rabbits used in this study were treated humanely according to the legal and ethical requirements.

2.3.4 Preparation of standard samples

A batch of *M. hyopneumoniae* bacterial culture with a titer of 5 $\times 10^9$ CCU/mL was divided into 500 μL/tubes and stored at -20 °C as standard samples. For testing, a tube was taken out, thawed, and diluted 2, 4, 8, 16, 32, 64, 128, and 256 times with PBS buffer to obtain a series of standard samples.

2.3.5 Development of the ic-ELISA

Microtiter plates were coated with 100 μL per well of the whole *M. hyopneumoniae* antigen (10 μg/mL) obtained by ultrasonic crushing in carbonate- bicarbonate buffer (0.05 M, pH 9.6) at 4 °C overnight. After the plates were washed with PBST (0.01 M PBS containing 0.1% Tween-20), each well was blocked with 200 μL of blocking buffer and then incubated for 2 h at 37 °C. After washing three times with PBST, 50 μL of the test sample and 50 μL of rabbit antisera of EF-Tu were added to the well together, and the plates were incubated for 1.5 h at 37 °C. After the plates were washed with PBST, they were incubated with 100 μL of diluted horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG at 37 °C for 1 h. After washing, 100 μL of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was added and incubated at 37 °C in the dark for 15 min. Finally, the reaction was terminated by adding 50 μL of 2 M H_2SO4. The absorbance was determined at 450 nm using a BioTek uQuant microplate reader (Bio-Tek, Winooski, VT, USA). The wells incubated without sample but with EF-Tu-antisera were defined as the positive control. The results were expressed as B/B0: B/B0 =(OD sample) / (OD positive). The OD sample (B) is the optical density of the well with standard or test samples, while the OD positive (B0) is the optical density of the well without the sample (Naoki *et al.*, 2014).

2.3.6 Optimization of the ic-ELISA

Different conditions were optimized for the ic-ELISA: different blocking buffer (5% skimmed milk, 5% bovine serum albumin (BSA), 1% gelatin, or 1% casein), coating concentration (0.625 μg/mL, 1.25 μg/mL, 2.5 μg/mL, 5 μg/mL, 10 μg/mL, or 20 μg/mL), dilution ratio of polyclonal antibody against EF-Tu (1:2000, 1:5000, 1:10000, or 1:20000), diluent (PBS, PBST, 0.1% gelatin, 1% gelatin, Dulbecco’s modified Eagle’s medium

(DMEM), or KM2 medium), sample incubation time (1, 1.5, 2, or 3 h), dilution ratio of secondary antibody (1:2000, 1:5000, 1:10000, or 1:20000), secondary antibody incubation time (20, 30, 45, or 60 min), and chromogenic time (10, 15, 20, or 30 min). Each parameter was optimized so that the B0 value was close to 1, the B/B0 value of the undiluted standard sample (5×10^9 CCU/mL) was close to 0.2, and the linear range was wide. Using the optimized conditions, a standard curve was drawn with the dilution factors as the x-axis, and B/B0 as the y-axis. The data in the linear range of the standard curve were analyzed by log-linear regression.

2.3.7 Determination of the reproducibility of the ic-ELISA

Ten batches of different samples were tested three times in one or three batches of the ic-ELISA kits. The intra-assay coefficient of variation (CV, CV =standard deviation (SD) / mean ×100) and the inter-assay CV were calculated, respectively.

2.3.8 Preparation and continuous detection of cultures at different growth stages

Ten milliliters of strain NJ were inoculated into 90 mL of KM2 medium. Three batches of culture were grown at 37 °C statically for 6 days until they reached the death phase (termed NJ-1, NJ-2, and NJ-3). Samples were taken at different times for pH, CCU, and ic-ELISA titer assessments. For the convenience of comparison, the ic-ELISA data were expressed as 1/(B/B0). Titers were determined as the means of three measurements. Pearson correlation analysis was performed on the CCU vs. ic-ELISA data obtained from the samples in the logarithmic growth phase.

2.3.9 Application of the ic-ELISA in the vaccine manufacturing process

An in-house reference vaccine sample was provided by the Jofunhwa Biotechnology Co., Ltd., Nanjing, China. Three batches of cultures (noted as test vaccine sample batches 1, 2, and 3) were produced by the Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Nanjing, China. Both the reference sample and test samples were measured using the ic-ELISA. The x values (dilution factors) were calculated using the established formula with the measured y values (B/ B0). The relative potency (RP) for each test sample was calculated as x (reference)/x (test). If the RP ≥1.0, the test sample was judged

to meet the quality standard. The samples were also tested using CCU assays for comparison.

2.3.10 Comparison of the ic-ELISA and CCU result on the cultures with different titers

To analyze the relationship between ic-ELISA and CCU titers, 20 samples of strain NJ were produced independently with 20 different batches of KM2 medium containing 5 different batches of swine serum each. The samples were harvested at the maximal growth phase (end of logarithmic growth) were measured using both assays. A linear regression equation was set taking lg CCU/mL as the x-axis and 1/(B/B0) as the y-axis.

2.3.11 Data analysis

All statistical analyses were carried out using GraphPad Prism soft-ware (Version 8.0.1, GraphPad Software Inc., La Jolla, CA, USA). The data in the linear range of the standard curve of ic-ELISA were analyzed using log-linear regression with dilution factors as the x-axis, and B/B0 as the y-axis. Pearson correlation analysis was performed on the CCU and ic-ELISA data obtained from the samples in the logarithmic growth phase of three batches of cultures. Linear regression (lg CCU/mL as the x-axis and 1/(B/B0) as the y-axis) was used to analyze the relationship between the ic-ELISA and CCU data of 20 batches of cultures harvested at the end of the logarithmic growth phase.

2.4 Results

2.4.1 Establishment and optimization of the ic-ELISA assay

The antigen used the whole *M. hyopneumoniae* protein as the coating protein and anti-EF-Tu polyclonal antibodies as the detecting antibodies, an ic-ELISA was established. The optimal concentrations and dilution ratios of the coating antigen, polyclonal antibodies against EF- Tu, and HRP-conjugated goat anti-rabbit IgG were 10 μg/mL, 1:10,000, and 1:10,000, respectively. Meanwhile, the optimal blocking solution was 1% gelatin. The optimal sample incubation time, the incubation time of secondary antibody, and the chromogenic development time were 1.5 h, 1 h, and 15 min, respectively. A standard curve of the ic- ELISA method was obtained based on a serial dilution of standard samples, with the value of the dilution factors as the x-axis and B/B0 ((OD sample)/(OD positive)) as the y-axis (Fig. 2.1A). The data in the linear range of the standard curve, between dilution factors 2 and 128 times, were analyzed using log-linear regression. A coefficient of determination (R2) of 0.9904 was obtained, and the regression equation was y =0.0963log2(x) +0.1676 (Fig. 2.1B).

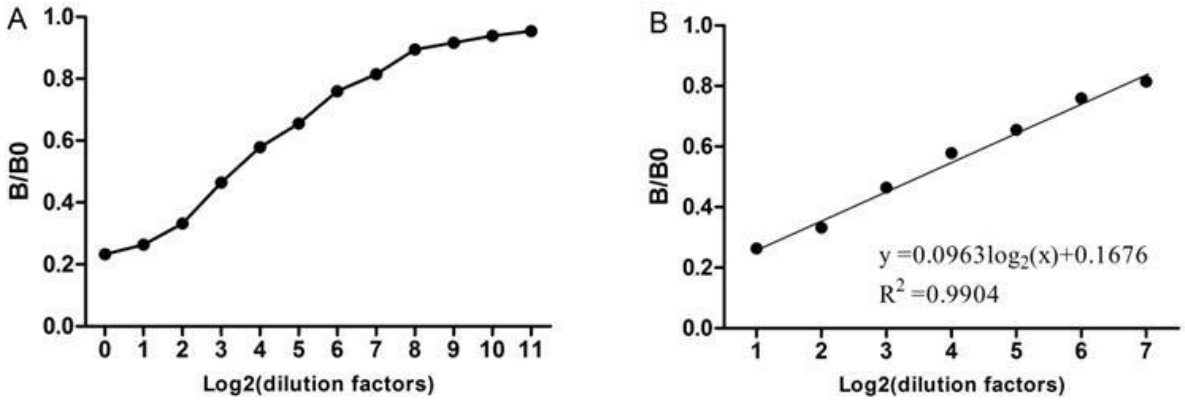

Figure 2. 1 The standard curve and regression equation obtained from the data of standard *M. hyopneumoniae* samples detected by the optimized ic-ELISA. (A) The curve constructed taking the dilution factors as the x-axis and B/B0 as the y-axis. (B) The result of log-linear regression based on the data in the linear range in the standard curve.

2.4.2 Determination of the reproducibility of the ic-ELISA

Ten batches of different samples were tested three times in one or three batches of ic-ELISA kits. The results of the reproducibility study are shown in Table 2.1. The assay showed an adequate reproducibility (less than 10%) (Bernhard *et al.*, 2021; Zuo *et al.*, 2021), with an intra-assay CV in the range of 1–7%, and an inter-assay CV lower than 10%.

Table2. 1 Reproducibility analysis of the ic-ELISA test.

	Sample No.										Mean
	1	2	3	4	5	6	7	8	9	10	
Intra-assay CV (%)	2.05	3.46	2.87	4.56	5.94	1.68	5.97	3.52	6.85	3.15	4.01
Inter-assay CV (%)	4.68	5.09	4.63	2.93	8.11	8.71	3.25	8.77	7.16	8.02	6.14

Ic-ELISA, indirect competitive enzyme linked immunosorbent assay; CV, coefficient of variation.

2.4.3 Continuous detection of cultures at different growth stages

The established ic-ELISA method and the traditional CCU method were used to detect the *M. hyopneumoniae* cultures at different culture stages. Simulating the production process of the vaccine, the seed was inoculated at a ratio of 1:10 into KM2 medium, and observed for 6 days. During these different growth stages, samples were taken for CCU and ic- ELISA assessments, and their pH values were also measured. The results of three different batches of culture (NJ-1 to NJ-3) are shown in Fig. 2.2. Throughout the culture cycle, the pH continued to drop from around 7.46 to 5.73 by 96 h of incubation, and was then maintained at about 5.7. The CCU increased slowly at first, followed by an exponential rise, and then a rapid decline. The inflection point of the CCU curves of all the three batches appeared at 48 h, when the pH was around 6.7. The trend of the curve of the ic-ELISA was similar to that of the CCU assay. However, notably, the time to reach the peak in the ic-ELISA curve was 60 h, which was relatively later than that in the CCU curve. After reaching the peak, the amount of antigen remained constant for a while before it began to decline significantly (after 84 h), unlike the CCU curve, which dropped immediately and sharply after reaching the peak.

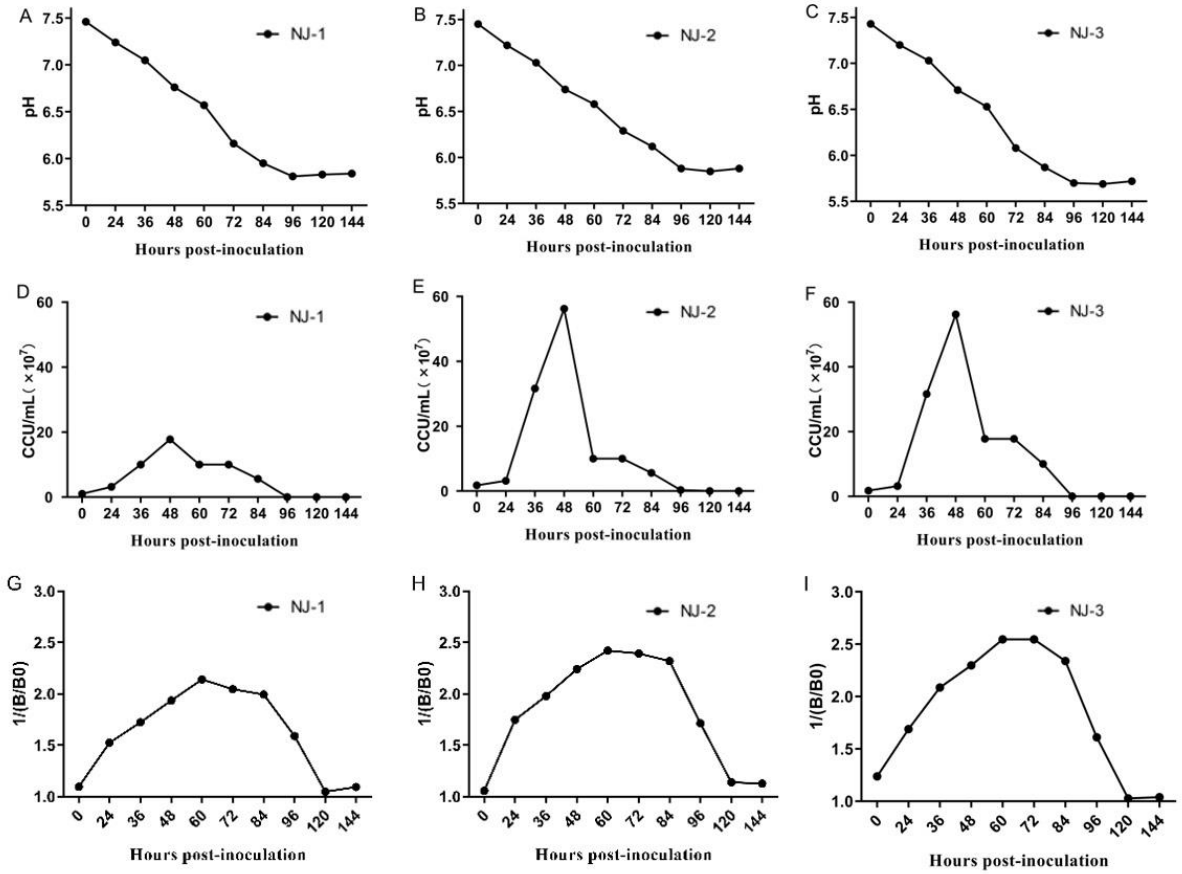

Figure 2. 2 pH, CCU, and ic-ELISA determination of continuous cultures of *M. hyopneumoniae*. Three batches were grown at 37 °C statically until the death phase was reached (NJ-1 to NJ-3). During these growth periods, samples were taken for pH (A-C), CCU (D-F), and ic-ELISA (G-I) assessments; titers were determined as the means of three measurements.

During the logarithmic growth phase (24–48 h), the change trend of the CCU value of the culture was similar to that of the antigen amount determined using ic-ELISA. Pearson's correlation analysis was carried out to preliminarily evaluate the relationship between them on three batches of cultures. As shown in Table 2.2, significant associations were found between the results of CCU and ic-ELISA for all three batches (P <0.05).

Table 2. 2 Correlation between CCU and ic-ELISA assays considering the data from the logarithmic growth phase of three batches of *M. hyopneumoniae* cultures.

Cultures	r	R^2	*P*
NJ-1	0.9947	0.9895	< 0.01
NJ-2	0.9637	0.9287	<0.05
NJ-3	0.9628	0.9271	<0.05

Ic-ELISA, indirect competitive enzyme linked immunosorbent assay; CCU, color changing units; r, Pearson correlation coefficient; R^2, coefficient of determination.

2.4.4 Application of the ic-ELISA during the vaccine production process

In the vaccine production process, the company usually sets up an in- house reference vaccine sample according to the vaccine quality standard. RP values obtained by comparing the test samples with the reference sample are used to judge whether the test samples meet the quality standard. In the present study, three batches of *M. hyopneumoniae* strain NJ were cultured as test samples and were harvested when the pH reached 6.7. The amounts of antigen of the test samples and the reference sample were determined using ic-ELISA. The RP values of Batch-1 and Batch-3 were both above 1.0, indicating that they met the quality standard, while that of Batch-2 was lower than 1.0, indicating that this batch did not meet the quality standard (Table 2.3). At the same time, the CCU values of the batches of cultures were also measured, and the results were consistent with those of the ic-ELISA test.

Table 2. 3 The RP of ic-ELISA *vs*. CCU data for three batches of *M. hyopneumoniae* cultures.

Cultures	RP of ic-ELISA	CCU
Batch-1	1.27 ± 0.06	9.00 ± 0.00
Batch-2	0.85 ± 0.08	8.67 ± 0.58
Batch-3	1.55 ± 0.14	9.67 ± 0.58
Reference product	—	9.00 ± 0.00

RP, relative potency; ic-ELISA, indirect competitive enzyme linked immunosorbent assay; CCU, color changing units.

2.4.5 Relationship between ic-ELISA and CCU assays

The high correlation between CCU and ic-ELISA results in the logarithmic growth phase prompted us to further study the proportional relationship between the ic-ELISA results and the CCU results, with the aim of quickly calculating the CCU values using the ic-ELISA results. Twenty batches of *M. hyopneumoniae* strain NJ were cultured with different batches of KM2 medium and harvested when the pH reached 6.7. The titers were determined using both the CCU and ic-ELISA methods. A linear relationship was established taking the lg CCU/mL as the x-axis and 1/(B/B0) of the ic-ELISA value as the y-axis. The obtained formula was y =0.876 x - 5.7939, with an R2 value of 0.9809 (Fig. 2.3).

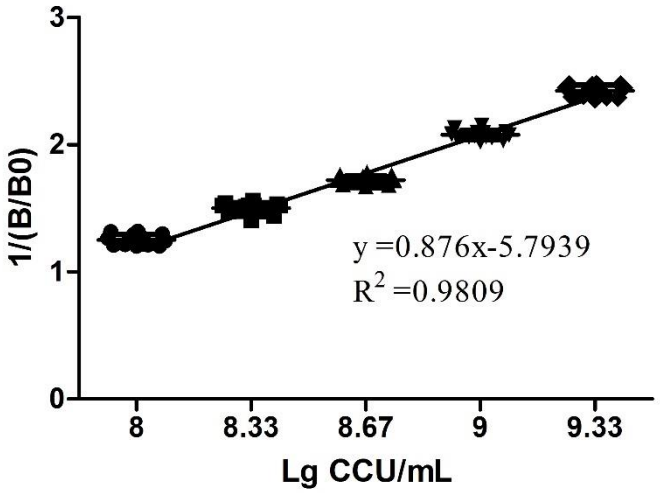

Figure 2. 3 The correlation between the CCU assay and the ic-ELISA assay. Twenty samples were harvested at the maximal growth phase (the end of logarithmic growth) and measured using both CCU and ic-ELISA assays. A linear regression equation was established taking the lg CCU/mL as the x-axis and 1/(B/B0) ((ODsample)/(ODpositive)) value of ic-ELISA as the y-axis.

2.5 Discussion

Researchers have attempted to develop alternative methods to determine the amount of *M. hyopneumoniae* rapidly, due to the limitations of the CCU and CFU methods. Calus *et al.* used an ATP assay to determinate the titer of *M. hyopneumoniae* culture. The technique is based on an enzymatic reaction in which cellular ATP reacts with D- luciferin and oxygen under the control of luciferase. This reaction produces a light signal with an intensity proportional to the amount of ATP and can be quantified using a luminometer. The results showed that CCU and ATP titers were linked strongly and linearly during the logarithmic phase. The ATP assay is faster, more accurate, and cost efficient compared with the CCU assay (Calus *et al.,* 2010). Assunc`ao *et al.* evaluated the potential application of flow cytometry to determine the growth rates of *M. hyopneumoniae* in a broth medium, and the data showed that flow cytometry is a very useful, practical, and fast technique to study the growth rates of *M. hyopneumoniae* in broth medium (Assuncao *et al.*, 2005). Morante *et al.* compared different methods (ATP, CCU, CFU, and qPCR) to determine *M. hyopneumoniae (*Garcia-Morante *et al., 2018)*. The results showed that all three strain cultures showed the same growth phases as well as similar maximal titers within a particular technique, except for CFU. During the logarithmic phase of growth, the CCU, ATP, and qPCR titers were strongly and linearly associated, and the correlations between the techniques could be reliably established. However, the methods mentioned above have their own merits and disadvantages. Similar to the CCU/CFU method, the ATP method detects the metabolism of live bacteria, which is suitable to assess live, but not dead bacteria in culture. qPCR mainly detects the content of DNA; however, the correlation between the protein content and the DNA content needs to be evaluated.

Protein antigen is the core element for an inactivated vaccine. The accumulation of proteins and changes in bacterial activity are not totally synchronized; therefore, detection methods targeting proteins, such as ELISA, are more direct and suitable than methods that detect live bacteria or nucleic acid for the quality and quantity evaluation of inactivated vaccines. In this study, an EF-Tu protein based ic-ELISA assay was established, which provides a rapid method for determination of antigen concentration in *M. hyopneumoniae* inactivated vaccine.. Flow cytometry was used to detect if EF-Tu expressed stably on the surface of the *M. hyopneumoniae* cells at different growth stages (Appendix, Sup. Fig. 1). The quantity of the surface-exposed EF-Tu was verified stable during 0–60 h after seeding. It

indicated that freshly harvested *M. hyopneumoniae* cells, usually harvesting at the end of logarithmic growth phase with a pH around 6.7 (48 h in this study), were suitable for EF-Tu-based quantitation. However, as coming to the very late growth stage, the quantity of the surface-exposed EF-Tu at 72 h (24 h after reaching the peak of CCU) showed significant decreased tendency. This may be the result of decline state of *M. hyopneumoniae* cells and should be avoided for EF-Tu-based quantitation. The ic-ELISA was also applied to a different *M. hyopneumoniae* strain (avirulent reference strain J) to confirm its validation and stability on different strains (Appendix, Sup. Fig. 2). The curves obtained from these two different strains were very similar using the protein of the respective strain as coating antigen. Moreover, the cross detection (taking the protein of each other as coating antigens) revealed consistent results between them as well.

Since the EF-Tu gene is conserved among mycoplasma species, the EF-Tu-based ic-ELISA is suitable for quantification of pure mycoplasma cultures, but not for the purpose of identification of clinical samples or isolated mycoplasmas. However, its high conservation also provides a possibility to be used in other mycoplasma species. The surface localization of EF-Tu has been reported for various species of mycoplasmas (Balasubramanian *et al.*, 2009; Widjaja *et al.*, 2020). We tried to apply this method to another swine mycoplasma, *Mycoplasma hyorhinis*, using its own EF-Tu protein (Appendix, Sup. Fig. 3). Similar standard curve was obtained on *M. hyorhinis*, which supports its potential universal applicability in mycoplasma quantification. By analyzing the results of the CCU and ic-ELISA assays during the whole culturing process, we found that the CCU curve reached the in-flection point a little earlier than that of the ic-ELISA curve. When CCU reached its maximum value, ic-ELISA value was still rising, which indicated that the protein was still accumulating at this time. It reached its peak at 60 h, and was maintained for 12–24 h before declining sharply. The results suggested that the cultures used to produce inactivated vaccines could be harvested a little later than those used to pro-duce live vaccines. More antigen could be obtained at about 12 h after the end of the logarithmic growth phase. The ic-ELISA assay is more accurate and convenient to determine the time to harvest the antigen of an inactivated vaccine compared with the CCU assay.

In the present study, we observed that the results of the CCU assay and the established ic-ELISA assay correlated positively during the logarithmic growth phase (Table 2.2). Therefore, it will be possible to calculate the CCU results rapidly using the ic-ELISA results,

if a quantitative relationship between them could be determined. However, during the entire culture cycle, the change in the CCU titer and amount of protein are not completely synchronized. For example, in the early stage of culture, there will be a considerable number of dead cells in the seed solution, and their protein components are relatively large; how-ever, their contribution to the CCU titer is relatively small. By the end of the culture process, when the bacteria begin to die, the protein antigen will accumulate for a while before it degrades. Considering that bacteria are generally harvested at the end of the logarithmic growth phase, we planned to use the cultures harvested at this specific time point for analysis. In the actual cultivation of *M. hyopneumoniae*, the time point of the end of the logarithmic growth phase cannot be determined using either turbidimetric or CCU measurements. According to the study, the pH value influences and reflects the growth state of mycoplasma directly; therefore, a pH indicator is usually added to the medium to determine the harvesting time by observing the degree of discoloration. We compared the relationship between pH and the CCU value (Fig. 2.2), which showed that when the pH reached 6.7, the CCU started to show an inflection point, which could be taken as the harvesting time point for the live bacteria culture. Thereafter, we prepared 20 batches of *M. hyopneumoniae* cultures grown in 20 batches of media, and harvested them when the pH reached 6.7. The CCU and ic-ELISA methods were used to detect the titer, and the results of these two assays were subjected to linear regression analysis to deduce a regression equation. Using the equation, the CCU titer could be calculated quickly based on the results of ic-ELISA. This would be convenient in situations where there is a need to determine the CCU quickly.

In summary, an ic-ELISA method with EF-Tu as the target protein was developed for the rapid detection of the amount of *M. hyopneumoniae*. It is suitable for antigen quantification during the production process of inactivated vaccines. At the same time, the statistical correlation was analyzed between the results of the CCU and ic- ELISA methods, based on which, we determined that the CCU titer could be calculated rapidly from the ELISA results.

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CHAPTER 3. COMPARATIVE STUDY ON THE INACTIVATION AND IMMUNOGENICITY OF MYCOPLASMA HYOPNEUMONIAE VACCINES PREPARED USING DIFFERENT INACTIVATORS

3.1 Abstract

The present study aimed to investigate the optimal inactivators and inactivation conditions for preparing inactivated vaccines of *Mycoplasma hyopneumoniae*. *M. hyopneumoniae* inactivation was performed using formaldehyde, thimerosal, β-propiolactone (BPL), and binary ethylenimine (BEI) and compared. The results showed that *Mycoplasma hyopneumoniae* was completely inactivated when incubated with 0.01% formaldehyde for 24 h or 0.02% formaldehyde for 12 h at 37℃, with 0.0008% thimerosal for 12 h at 37℃, with 0.02% BPL for 24 h or 0.1% BPL for 12 h at 4℃, or with 0.004% BEI for 24 h or 0.5% BEI for 12 h at 37℃. Next, the immunogenicity of *M. hyopneumoniae* after inactivation was evaluated by immunizing BALB/c mice. Mice were separately immunized with a high dose of inactivated mycoplasmas (10^6 CCU per dose) and a low dose of inactivated mycoplasmas (10^4 CCU per dose). In mice immunized with a high dose of mycoplasmas, *M. hyopneumoniae* vaccines inactivated with all inactivators led to high levels of serum IgG antibodies. However, in mice immunized with a low dose of mycoplasmas, only *M. hyopneumoniae* vaccines inactivated with formaldehyde and BEI led to significant levels of serum IgG antibodies. Among these groups, the antibody levels in the formaldehyde-inactivated vaccine group were higher than those in the other inactivated by other solutions. This study provides a reliable basis for inactivation during large-scale production of *M. hyopneumoniae* inactivated vaccines.

3.2 Introduction

Mycoplasma species cause chronic infection in pigs and are difficult to eliminate from pig farms. *Mycoplasma hyopneumoniae* is the key pathogen of porcine endemic pneumonia (EP) (Maes *et al.*, 2018; Simionatto *et al.*, 2013), a globally distributed respiratory disease that has a significant impact on the economy. The main symptom of the disease is a dry cough, accompanied by reduced growth performance. In addition, *M. hyopneumoniae* also contributes to the porcine respiratory disease complex (PRDC) through potentiation of respiratory diseases caused by viral pathogens such as porcine reproductive and syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) (Oh *et al.*, 2019).

Vaccination remains the most effective approach to control the infection caused by the pathogens (Maes *et al.*, 2018; Martinson *et al.*, 2018b; Nicholas *et al.*, 2009; Simionatto *et al.*, 2013). The commercial vaccines mainly include inactivated vaccine and live vaccine for *M. hyopneumoniae*. Inactivated vaccines consist of inactivated *M. hyopneumoniae* whole-cell preparations and adjuvants (Maes *et al.*, 2021a), are used worldwide. In order to obtain sufficient protection, adjuvants are often added in the inactivated vaccines to enhance the immune response of animals. The content of antigen and the type of adjuvant are the key factors to the efficacy of inactivated vaccine. Inactivated vaccines are usually injected intramuscularly and induce the body to produce specific antibody against *M. hyopneumoniae*. Attenuated vaccines against *M. hyopneumoniae* are licensed for use only in Mexico and China (Feng *et al.*, 2013). In China, the attenuated vaccine (strain 168) was inoculated by intrapulmonary injection, while the attenuated vaccine used in Mexico was inoculated by nasal drip. Local mucosal immunity and humoral immunity could be induced in the respiratory tract by intrapulmonary and nasal inoculation of the attenuated vaccine.

Ensuring vaccine safety and effectiveness is the chief objective during vaccine development. Generally speaking, the virulence of strain is higher, the immunogenicity is better. Therefore, the strains used to prepare inactivated vaccines are the high virulent strains generally. In the production of inactivated vaccines, complete inactivation of pathogens is a crucial step to eliminate the risk of infection and ensure vaccine safety. The outcome of inactivation is closely associated with the inactivators used. It should be noted that the inactivation process may be accompanied by antigen damage, which may lead to different

degrees of reduction of vaccine immunogenicity. A good inactivator inactivates the pathogen completely while retaining good antigenicity. On the other hand, the safety of inactivators is also very important, which should be safe enough for animals or human beings within the dosage range. Therefore, identification of efficient and safe inactivators is an important step in the development of inactivated vaccines.

At present, the inactivators used for inactivation of *Mycoplasma* include formaldehyde, thimerosal, β-propiolactone (BPL), and binary ethylenimine (BEI) (David *et al.*, 2010; Gong *et al.*, 2020a; Mwirigi *et al.*, 2016b; Tamiya *et al.*, 2020; Yan-Wu Wei, 2020). For most human and animal vaccines, thimerosal is often used in anti-mildew and anti-corrosion treatment of biological products, which has a certain inactivation effect on fungi, bacteria, and viruses (Clements, 2004; Golos and Lutynska, 2015). BPL achieves microbial inactivation by affecting the structure of microbial DNA or RNA. In general, the pathogens were inactivated by BPL at 4 ℃, and then incubated 2h at 37 ℃ to remove the residual BPL, which can eliminate the residual BPL in the inactivated antigen (Goncalves *et al.*, 2014; Kamaraj *et al.*, 2008). BEI is a derivative of ethylene imine, which eliminates viral infectivity without damaging the viral capsid proteins. When the pathogens were inactivated completely by BEI, 2% sodium thiosulfate needs to be added to block the inactivation. Formaldehyde is one of the most widely used conventional inactivator. When treated with formaldehyde, bacteria or viruses lose their infectivity owing to the alkylation of proteins and nucleic acids. In the present study, the inactivation effects of formaldehyde, thimerosal, BPL, and BEI on *M. hyopneumoniae* and the immunogenicity of antigens after inactivation were determined and compared to identify a suitable inactivator for producing *M. hyopneumoniae* inactivated vaccines.

3.3 Materials and methods

3.3.1 *M. hyopneumoniae* strains and culture conditions

M. hyopneumoniae strain NJ, isolated from Nanjing in China, was cultured in KM2 liquid medium containing 20% (v/v) pig serum at 37℃ (Liu *et al.*, 2013). The *Mycoplasma* titer in the cultures was determined using a color-changing unit (CCU) assay, which is based on the indicator in the medium changing its color to yellow. In brief, ten-fold serial dilutions (up to 10^{-11}) of *Mycoplasma* cultures were prepared using KM2 medium. The tubes were incubated at 37℃ and observed for 2 weeks. The CCU titer was determined from four repeated measurements and was calculated per millilitre culture as 10^{y}, and y is the mean of the number of tubes that turned yellow by acidification due to *M. hyopneumoniae* growth in each of the repeated measurements (Calus *et al.*, 2010; Garcia-Morante *et al.*, 2018).

3.3.2 Inactivation of *M. hyopneumoniae*

M. hyopneumoniae strain NJ was grown in KM2 liquid medium at 37℃ for 36–48 h. To test the inactivation of mycoplasmas, 10 mL of actively growing mycoplasma cultures treated with different concentrations of formaldehyde, thimerosal, BPL, and BEI as shown in Table 1 and a control without any inactivator was included. *Mycoplasma* cultures treated with formaldehyde, thimerosal, or BEI were kept at 37℃ (Goldstein *et al.*, 1970; Takada *et al.*, 2003; Wenzel *et al.*, 1977), whereas the cultures treated with BPL were kept at 4℃ (Anjeanette Roberts, 2010). All treated cultures were incubated for 12 h and 24 h and thereafter assay for residual live mycopslama (see section 3.2.3). And the end of the inactivation, samples inactivated with BEI were hydrolyzed by adding sterile sodium thiosulfate solution to a final concentration of 2% (Christianson *et al.*, 1980). Inactivated samples were stored at 4℃ until further use.

Table 3. 1 Chemical agents, concentrations, and inactivation conditions used in the study

Chemical	Final concentration (%)	Inactivation time (h)	Inactivation temperature (°C)
Formaldehyde	0.0025%, 0.005%, 0.01%, 0.02% (v/v)	12, 24	37℃
Thimerosal	0.00016%, 0.0008%, 0.004%, 0.02% (w/v)	12, 24	37℃
Beta-propiolactone	0.004%, 0.02%, 0.1%, 0.5% (v/v)	12, 24	4℃
Binary ethylenimine	0.004%, 0.02%, 0.1%, 0.5% (v/v)	12, 24	37℃

3.3.3 Detection of *M. hyopneumoniae* activity after inactivation

To assess the residual live mycoplasmas after treatment with inactivator, a 1 mL sample was taken from the cultures at 12 h or 24 h and centrifuged at 12000 × *g* for 20 min at 4°C. The precipitate was resuspended in 1 mL of phosphate-buffered saline (PBS) followed by determining the *M. hyopneumoniae* titer using the CCU assay (David *et al.*, 2010). The color of the *Mycoplasma* culture was observed every day. If the color of the cultures did not change after two weeks, the samples were passaged in KM2 liquid medium at a ratio of 1:10 every 7 days for three generations. If the color of the *M. hyopneumoniae* cultures did not change during the three passages, then the inactivation was considered to be complete.

3.3.4 Immunogenicity evaluation of the inactivated *M. hyopneumoniae* in mice

On the basis of the results of the inactivation test, the lowest concentration and shortest incubation time for each inactivator were selected to prepare the *M. hyopneumoniae* vaccines. The inactivated *M. hyopneumoniae* cultures were diluted to 3.5×10^7 CCU/mL or 3.5×10^5 CCU/mL and mixed with Tween-80 (4%, v/v). Vaccines were prepared by emulsifying the aqueous phase with the Marcol white mineral oil adjuvant at 10:25 (v/v). Six–eight-week-old, male BALB/c mice (Institute of Comparative Medicine, Yangzhou University) were inoculated intramuscularly with 0.1 mL of the *M. hyopneumoniae* vaccines, 8 mice per group (containing 10^6 CCU or 10^4 CCU per dose). Mice immunized with PBS were used as negative control. The immunization was repeated after 14 days.

3.3.5 Detection of antibodies induced by vaccine immunization

Serum samples were collected at 0, 7, 14, and 21 days and 0, 14, 28, and 42 days after the second inoculation from mice immunized with 10^6 and 10^4 CCU of mycoplasmas, respectively. Antibodies against *M. hyopneumoniae* were detected with an in-house developed indirect ELISA method. In brief, *M. hyopneumoniae* cells were collected by centrifugation at 12000 × *g* for 20 min; the pellet was washed thrice and resuspended in PBS. After lysis by ultrasonication, the supernatant was collected, and its protein concentration was determined using the BCA kit. The supernatant was diluted to 5 μg/mL with carbonate–bicarbonate buffer (pH 9.6) and used to coat 96-well ELISA plates overnight at 4°C. After blocking with 5% bovine serum albumin (BSA), each well was incubated with 100 μL of serum sample (1:100, in PBS containing 5% BSA) for 30 min at 37°C, followed by the addition of 100 μL horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Boster, China; 1:10,000, in PBS containing 5% BSA). After washing, the reaction was visualized by incubation with tetramethylbenzidine-hydrogen peroxide (TMB, Beyotime, China) substrate for 10 min at 25°C. The optical density (OD) of the solution was measured at 450 nm using a BioTek uQuant microplate reader (Bio-Tek, Winooski, VT, USA).

3.3.6 Statistical analysis

Data was expressed as the mean ± standard deviation (SD). Repeated-measures ANOVA was used to analyse the differences in antibody levels among groups. $p < 0.05$ was set as the threshold for statistical significance.

3.4 Results

3.4.1 Inactivation of *Mycoplasma hyopneumoniae*

The inactivation of *M hyopneumonia*e by formaldehyde, thimerosal, BPL, and BEI at different concentrations was evaluated as *M. hyopneumoniae* inactivation agents for the production of bacterin. The bacterium was cultured to mid-log phase at a bacterial titer of 10^8-10^9 using the CCU assay. Different concentrations of inactivator were added to *M. hyopneumoniae* cultures and incubated for 12 or 24 h at 37℃ (4℃ for BPL), then samples were taken at 12 and 24 h for the CCU assay. As shown in Figure 3.1, the titer of *M. hyopneumoniae* cultures (CCU/mL) after 12 or 24 h incubation decreased as the inactivator concentration increased. Complete inactivation was achieved by all four inactivator. When 0.01% of formaldehyde was added to *M. hyopneumoniae* cultures for 24h, or 0.02% of formaldehyde was added to *M. hyopneumoniae* cultures for 12h, *M. hyopneumoniae* did not grow. The results showed that complete inactivation was achieved at a concentration of 0.01% at 24 h and 0.02% at 12 h. In the same way, *M. hyopneumoniae* was completely inactivated by thimerosal at a concentration of 0.0008% at 12 h, by BPL at a concentration of 0.02% at 24 h and 0.1% at 12 h, and by BEI at a concentration of 0.004% at 24 h and 0.5% at 12 h.

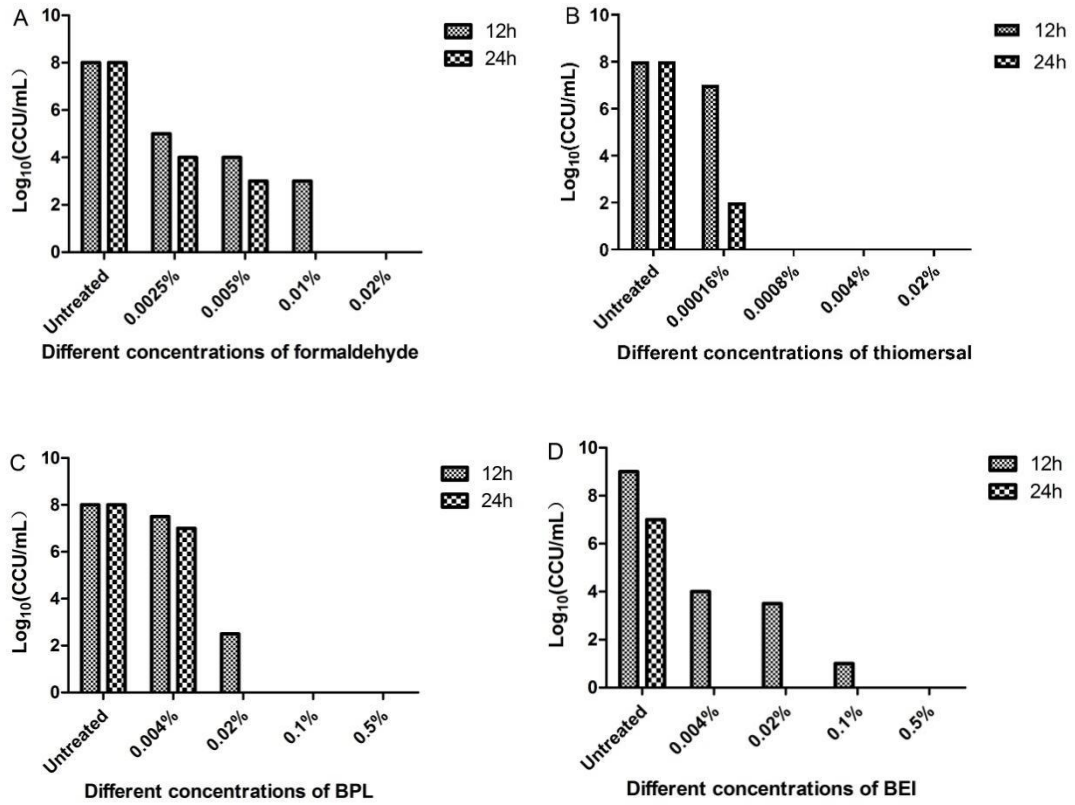

Figure 3. 1 Dynamics of *Mycoplasma hyopneumoniae* inactivation performed using four different inactivator. *Mycoplasma hyopneumoniae* cultures were treated with different concentration of formaldehyde, thimerosal, BPL, and BEI at 37℃ (4℃ for BPL) for 12 h or 24 h. A control without any inactivator was included. The titers of the cultures after treatment were determined by the CCU assay.

3.4.2 Immunogenicity evaluation of inactivated *Mycoplasma hyopneumoniae* in mice

It is important for a vaccine to be immunogenic. A good inactivator should inactivate the pathogen while retaining the immunogenicity as much as possible. Therefore, the immune responses of the mice received immunizations with vaccines inactivated by different inactivators were detected. The intensity of humoral immune response is also closely related to the immune dose of the vaccine. High and low immunization doses were chosen to compare the influence of inactivators on the immunization. On the basis of the results above, *M. hyopneumoniae* was inactivated using different inactivators at the lowest concentration and for the shortest incubation time demonstrated to be effective (0.01% formaldehyde and 0.004% BEI for 24 h and 0.0008% thimerosal for 12 h at 37℃ and 0.02% BPL for 24 h at

4℃). High-dose (10^6 CCU *M. hyopneumoniae* per dose) and low-dose (10^4 CCU *M. hyopneumoniae* per dose) inactivated vaccines were prepared and used to immunize BALB/c mice. The IgG antibodies in serum were detected to evaluate the immunogenicity of the vaccines. All vaccinated mice produced significant quantities of serum IgG antibodies at 7 days post the second immunization with a high dose of antigens ($p < 0.05$; Tables 3.2). The antibody levels of mice immunized with formaldehyde-inactivated vaccines were significantly higher than those of mice immunized with vaccines inactivated by other inactivators ($p < 0.05$; Table 3.2). In mice inoculated with a low dose of antigens, only mice that received vaccines inactivated using formaldehyde or BEI showed significantly different antibody levels ($p < 0.05$) compared with mice from the control group. The IgG levels in the formaldehyde group were higher than those in the BEI group ($p < 0.05$). Mice immunized with thimerosal- or BPL-inactivated vaccines did not show a significant increase in antibody levels compared with mice from the control group ($p > 0.05$).

Table 3. 2 Serum antibody levels of mice immunized with *Mycoplasma hyopneumoniae* inactivated vaccines prepared using different inactivators

Days post second immunization	The OD value in different groups				
	Control	**Formaldehyde**	**Thimerosal**	**BPL**	**BEI**
10^6 CCU per dose					
D0	0.12±0.00	0.15±0.02	0.14±0.02	0.14±0.01	0.14±0.02
D7	0.11±0.00	0.86±0.23	0.69±0.22	0.61±0.25	0.66±0.15
D14	0.12±0.00	0.69±0.21	0.60±0.18	0.58±0.13	0.61±0.13
D21	0.11±0.00	0.66±0.16	0.57±0.11	0.52±0.15	0.52±0.12
Statistical difference*	a	b	c	c	c
10^4 CCU per dose					
D0	0.10±0.01	0.17±0.06	0.10±0.01	0.10±0.02	0.13±0.07
D14	0.13±0.04	0.65±0.35	0.18±0.19	0.12±0.02	0.35±0.39
D28	0.12±0.03	0.97±0.50	0.20±0.21	0.14±0.06	0.47±0.51
D42	0.12±0.02	0.91±0.52	0.27±0.28	0.16±0.09	0.50±0.59
Statistical difference*	a	b	a	a	c

*containing one same letter indicates no statistically significant difference between groups ($p > 0.05$); without any same letter indicates a statistically significant difference between groups ($p < 0.05$). BEI, binary ethylenimine; BPL, β-propiolactone; CCU, color changing units

3.5 Discussion

In recent years, the most commonly used inactivators for preparing inactivated *Mycoplasma* vaccines are formaldehyde and BEI. For example, *M. mycoides subsp.mycoides* was inactivated with 0.7% formaldehyde (Mwirigi *et al.*, 2016b), *M. pneumoniae* was inactivated with 0.16% formaldehyde (Tamiya *et al.*, 2020), *M. hyorhinis* was inactivated with 0.2% formaldehyde (Yan-Wu Wei, 2020) or BEI (Martinson *et al.*, 2018b), and *M. synoviae* was inactivated with BEI (Gong *et al.*, 2020a).

In the present study, the inactivation of *M. hyopneumoniae* was performed using formaldehyde, thimerosal, BPL, and BEI and compared. The results showed that *M. hyopneumoniae* was completely inactivated by treatment with 0.01% formaldehyde for 24 h or 0.02% formaldehyde for 12 h at 37℃ and treatment with 0.004% BEI for 24 h or 0.5% BEI for 12 h at 37℃. These findings are consistent with the results that *M. hyorhinis* was completely inactivated by 0.001M (0.0067%) BEI (Christianson *et al.*, 1980). A 2010 study found that 22 mycoplasma species examined were completely inactivated within 3–24 h at room temperature in mycoplasma media containing 0.2% (66.60 mM) formaldehyde and 0.1% (13.87 mM) BPL (David *et al.*, 2010). Our results showed that 0.01% formaldehyde, 0.02% and 0.1% BPL completely inactivated *M. hyopneumoniae*, respectively. The optimal inactivation concentration identified for formaldehyde in the present study was relatively lower than that reported by David (David *et al.*, 2010). It is likely that the inactivation temperature used in the present study (37℃) was higher than that used in their study (room temperature).

Many studies have been performed to explore new inactivators for producing inactivated *Mycoplasma* vaccines. A 2015 study reported an innovative approach for inactivation of *Mycoplasma gallisepticum* using 1,5-iodonaphthyl azide (INA), which suggests that the hydrophobic, photoinducible alkylating agent INA inactivates *M. gallisepticum* but preserves its surface lipoproteins and thus has the potential to be used as a general approach for the *Mycoplasma* inactivation for vaccine development (Atalla *et al.*, 2015).

Immunogenicity is the core aspect of a vaccine; however, inactivation may cause some damage to immunogenicity. Generally, inactivators destroy bacterial or viral nucleic acids or proteins, so that the antigen is not as complete as an untreated antigen. Therefore, it is crucial

to explore the appropriate inactivators and optimal inactivation time and temperature for a specific vaccine. In general, with the increase in inactivator concentration and incubation temperature, the inactivation efficiency increases, and the required inactivation time decreases. A good inactivator should be able to inactivate the pathogens at a low concentration, in a short time, and at a low temperature while ensuring vaccine safety and efficacy. In the present study, the factors concentration and dose were comprehensively considered to optimize the inactivation conditions for four different inactivators. Immunization in mice was performed to assess the effect of the inactivation conditions on antigen immunogenicity. Formaldehyde was considered ideal for inactivating *M. hyopneumoniae* because the antibody levels of mice in this group were significantly higher than those in other groups in both high-dose or low-dose immunization experiments. In addition, antibody levels in the BEI group were also significantly higher than those in the thimerosal and BPL groups in the low-dose immunization experiment. The immunogenicity of *M. hyopneumoniae* are best maintained after formaldehyde inactivation. It suggests that formaldehyde is a suitable inactivator for developing combination vaccines against *M. hyopneumoniae*. As early as 1992, there was reported that the pigs were inoculated intramuscularly with formalin-inactivated *M. hyopneumoniae* in adjuvant and the vaccinated groups showed some protection when challenged (Weng *et al.*, 1992).

A few studies have shown that formaldehyde, a conventional inactivator, leads to some damage in antigens. In a study, *M. mycoides subsp. mycoides* culture was inactivated by adding 0.7% (v/v) of formaldehyde or by heat inactivation in a water bath at 56°C for 30 min, and antibody responses following vaccination were measured using the complement fixation test. The results showed that the antibody responses following the formaldehyde-inactivated vaccine was observed in 5/7 (71%) of the animals, which was lower than that observed in the heat inactivation group (9/9, 99%) (Mwirigi *et al.*, 2016b). However, in the present study, formaldehyde inactivation was found to be superior in terms of maintaining the immunogenicity of *M. hyopneumoniae*, which may be due to the lower concentration used (0.01%) than that in previous reports. A study showed influenza virus was inactivated with 0.1% BPL (v/v). However, hemagglutination and fusion ability of the inactivated samples were highly affected by BPL treatments, which indicated a certain of damage to the surface antigens (Herrera-Rodriguez *et al.*, 2019). Developed in 1927, thimerosal has been and is still being used as a preservative in some cosmetics, topical pharmaceuticals, and biological drug products, including vaccines. Until the beginning of this century, every tetanus-containing

vaccine in the US contained thimerosal, many at a concentration of 0.01%. But the use of thimerosal in vaccine reduced since increasing evidence revealed the adverse effects of thimerosal on humans, especially to infants and children (Geier *et al.*, 2015). Furthermore, detrimental effect of thimerosal on vaccine antigens has also been described, which might reduce the vaccine potency (Geier *et al.*, 2015; Sawyer *et al.*, 1994).

Notably, the difference in immunogenicity impairment among various inactivators was not significant in the high-dose immunization experiment. This is because the vaccine can still effectively stimulate the immune system and induce a strong immune response when the antigen dose is high enough. However, with an insufficient antigen dose, the difference in immunogenicity was fully reflected. Nevertheless, using an appropriate inactivator would reduce the amount of antigen required, which would help the vaccine manufacturers to reduce their production costs.

3.6 Conclusion

In this study, the inactivation effects of four inactivators on *M. hyopneumoniae* were compared, and the optimal inactivation concentration and time of the four inactivators were identified. Next, the immunogenicity of the mycoplasmas after inactivation was evaluated by immunizing BALB/c mice. The results showed that *M. hyopneumoniae* were completely inactivated after incubation with 0.01% formaldehyde for 24 h at 37℃. The antibody levels in mice immunized with formaldehyde-inactivated vaccines of *M. hyopneumoniae* was significantly higher than those in mice immunized with vaccines inactivated by other three inactivators, which suggests that formaldehyde was the most suitable inactivator. The study provides a reference basis for the preparation of the single or combination vaccines against *M. hyopneumoniae*.

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CHAPTER 4. CONCLUDING REMARKS

4.1 Summary of Results, Discussion and Conclusion

This study aimed to solve two technical problems which are experienced in the process of *M. hyopneumoniae* vaccine production*;* the quantitative detection and inactivation method. An ic-ELISA method with EF-Tu as the target protein was established for the rapid detection of *M. hyopneumoniae*. Quantification of *M. hyopneumoniae* cultures at different growth stages using the ic-ELISA test showed a similar curve to that of the traditional color changing units (CCU) assay. In the inactivated vaccine production process, the cultures could be harvested later than that for the live vaccine, at about 12 h after the end of the logarithmic growth phase. Different batches of cultures were measured for their relative potency value compared with the in-house reference vaccine, which was used to determine whether the cultures met the antigen amount requirements for vaccine preparation. Therefore, it can be used as a quick quantification method during the production process of inactivated vaccines. At the same time, the statistical correlation was analyzed between the results of the CCU and ic-ELISA methods, based on which, the CCU titer can be calculated according to the ELISA result.

To explore the inactivation method of *M. hyopneumoniae*, the mycoplasma was inactivated using formaldehyde，thiomersal，β-propiolactone (BPL) and binary ethylenimine (BEI). Consequently, *M. hyopneumoniae* could be completely inactivated by all the four inactivators, with different concentrations and different incubating time period. The optimized inactivation condition was an incubation with formaldehyde at the concentration of 0.01% for 24 h at 37℃, or an incubation with thiomersal at the concentration of 0.0008% for 12 h at 37℃, or an incubation with BPL at the concentration of 0.02% for 24 h at 4℃, or an incubation with BEI at the concentration of 0.004% for 24 h at 37℃. Following inactivation, the immunogenicity of *M. hyopneumoniae* was evaluated by immunising BALB/c mice. The antibody levels in groups of the formaldehyde-inactivated vaccines were higher than that in the groups of other inactivators. The results also suggest that formaldehyde may be the best inactivator among the four inactivators. This study provided a reliable basis for inactivation during the large-scale production process of *M. hyopneumoniae* inactivated vaccines.

4.2 Limitations and Recommendations

In the production process of inactivated vaccine, it is necessary to quantitatively detect the starting concentration of the antigen during the preparation to ensure sufficient dose in the final product. Therefore, in this study, mycoplasma cultures were taken as the object for antigen quantitative detection. During antigen production, of mycoplasma will be inactivated as per developed process followed by formulation to prepare the vaccine. The addition of adjuvants, as well as the emulsion preparation, may affect the final quantification of vaccine. the follow-up study, it is necessary to verify the effectiveness of the antigen quantitative method in the detection of finished vaccine products.

The surface localization of EF-Tu has been reported for various species of mycoplasmas. The ic-ELISA method was also used to detect the EF-Tu antigen of *M. hyorhinis*. As a result, similar standard curve was obtained on *M. hyorhinis.* It supports its potential universal applicability in mycoplasma quantification. However, it should be noted that the EF-Tu molecules of different species of mycoplasmas have high homology, therefore cross reactions is highly possible. Therefore, the EF-Tu based quantitative detection method is not suitable for combined vaccines of different mycoplasmas.

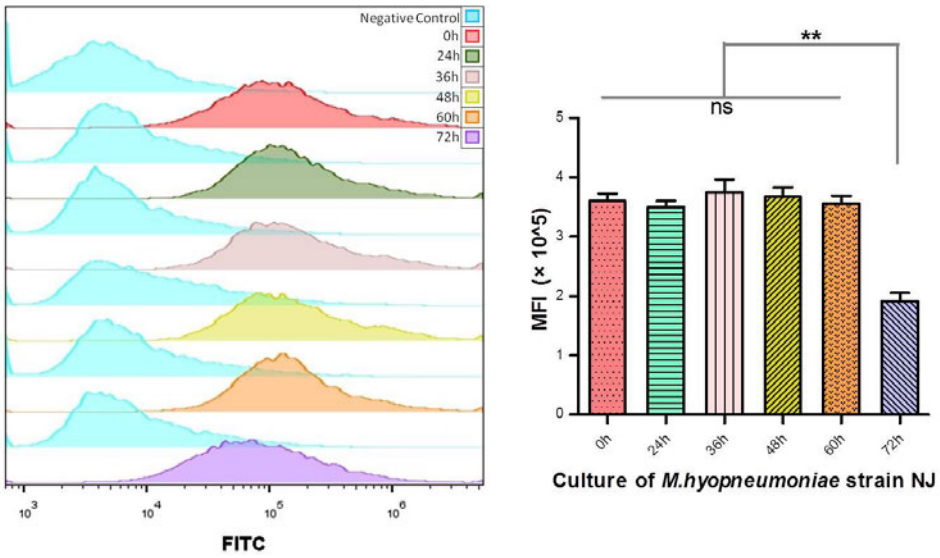
This study evaluated the immunogenicity of *M. hyopneumoniae* antigen with a goal to determine whether was there damage after inactivation. When evaluating the immunogenicity of *M. hyopneumoniae* antigen, it is best to use pigs as an animal model. However, considering the cost of study and the animal welfare, mice are often used as alternative model. Mice were immunized with vaccines inactivated by different inactivators and assayed for the serum antibodies. Formaldehyde was found to be the best inactivator for *M. hyopneumoniae*. Immune response includes humoral immunity and cellular immunity. Cellular immunity is also very important, but is relatively difficult to evaluate in a standardized way. In this study we only used humoral immunity to reflect the whole immune response. Furthermore, the species difference between mice and pig, as well as the difference between immune response and protection against challenge, should also be considered. Therefore, the effect of the *M. hyopneumoniae* vaccines inactivated by formaldehyde needs to be confirmed in pigs as an animal model.

Appendix

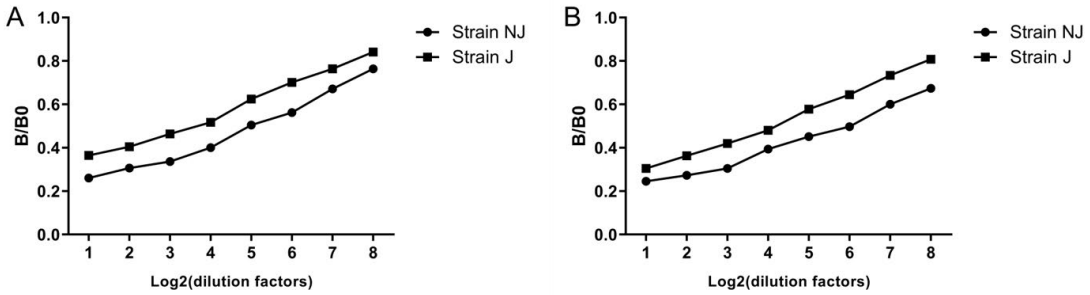
Supplementary Information Chapter 2

Sup. Table 1 Detection of secreted EF-Tu in supernatant of *M. hyopneumoniae* culture.

Test samples	RP
Supernatant	0.038 ± 0.00
Precipitate	1.20 ± 0.07



Sup. Fig. 1: Detection of EF-Tu on the surface of the *M. hyopneumoniae* cells at different growth stages. *M. hyopneumoniae* cells were harvested from cultures of 0 h, 24 h, 36 h, 48 h, 60 h, and 72 h respectively. Cellular surface EF-Tu was detected by flow cytometry with anti-EF-Tu serum. The pre-immune serum was used as a negative control. The assay was performed in triplicate, and the mean fluorescence intensity (MFI) of different samples was subjected to statistical analysis after subtracting the background value of samples incubated with pre-immune sera.



Sup. Fig. 2: Application of the ic-ELISA method on different *M. hyopneumoniae* strains. Microtiter plates were coated with the whole bacteria antigen of *M. hyopneumoniae* strain NJ (A) and strain J (B), respectively. Cultures of the two strains were serially diluted and detected using both the plate coated with the antigen of itself and the plate coated with the antigen of the other strain. Curves were drawn with the dilution factors as the x-axis, and B/B0 as the y-axis.

Sup. Fig. 3: Quantitative detection of *M. hyorhinis* by ic-ELISA based on *M. hyorhinis* EF-Tu. Microtiter plates were coated with the whole bacteria antigen of *M. hyorhinis.* A culture of *M. hyorhinis* was serially diluted and detected. The curve was drawn with the dilution factors as the x-axis, and B/B0 as the y-axis.