PORCINE DNA DETECTION IN COMMON TABLET FORMULATIONS AND EXCIPIENTS

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PORCINE DNA DETECTION IN COMMON TABLET FORMULATIONS AND EXCIPIENTS

by

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LIST OF ABBREVIATIONS

BP	British Pharmacopoeia
Ct	Cycle threshold
CTAB	Cetyltrimethylammonium bromide
Cy5	Cyanine 5
DNA	Deoxyribonucleic acid
FAM	6-carboxyfluorescein
HCl	Hydrochloric acid
Kg	Kilogram
MCC	Microcrystalline cellulose
Mg	Magnesium
mtDNA	Mitochondria DNA
Na	Sodium
NTC	No-template control
OD	Optical density
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
qPCR	Quantitative polymerase chain reaction
rpm	Revolutions per minute

LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
w/v	Weight over volume
w/w	Weight over weight
ng/ µl	Nanogram per microlitre

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PENGESANAN DNA PORSIN TERHADAP FORMULASI TABLET AM DAN EKSIPIEN

ABSTRAK

Permintaan pasaran ke atas produk farmaseutikal halal telah meningkat disebabkan oleh kesedaran pengguna Muslim terhadap produk halal. Dengan teknologi pengesahan DNA, eksipien dan produk akhir yang dicemari dengan kandungan porsin boleh dikesan dengan melakukan ujian kuantitatif tindak balas rantai polimerase (qPCR). Dalam kajian ini, tablet telah dipilih dan tiga jenis formulasi tablet telah dipilih; tidak bersalut, bersalut filem dan tablet pelepasan berterusan. Pengesanan DNA porsin telah dilakukan pada setiap eksipien digunakan dalam formulasi tiga jenis tablet. Templat kawalan positif dan negatif tablet tidak bersalut, tablet bersalut filem dan pelepasan berterusan tablet telah dihasilkan mengikut standard farmaseutikal untuk mengesan DNA porsin. Porsin dan bovin gelatin digunakan sebagai ejen pencemar dalam setiap eksipien dan sebagai pengikat dalam tablet kawalan positif dan negatif. Hasilnya, DNA porsin dikesan dalam semua eksipien yang dicemar dan tablet kawalan positif. Didapati bahawa larutan CTAB dapat memerangkap DNA dari matriks polisakarida yang terkandung dalam sodium starch glycolate dan prgelatinised starch. Keputusan menunjukkan bahawa kaolin yang terkandung dalam eksipiens salutan mempunyai tarikan yang tinggi kepada DNA dan dalam keadaan beralkali, interaksi yang kuat antara kaolin dan DNA boleh menjadi lemah. Hasil kajian menunjukkan bahawa porsin DNA masih boleh Berjaya diperoleh dan dikesan selepas proses pengeringan untuk pengeluaran tablet tidak bersalut. Oleh itu, pengesanan DNA porsin menggunakan kaedah ujian

qPCR boleh digunakan untuk mengesan kandungan porsin dalam tablet dan eksipien, apabila teknik pengekstrakan tertentu digunakan.

PORCINE DNA DETECTION IN COMMON TABLET FORMULATIONS AND EXCIPIENTS

ABSTRACT

Market demand on halal pharmaceutical products has increased due to the awareness of Muslim consumers towards halal products. With DNA verification technology, porcine content in adulterated excipients and final product can be detected by performing quantitative polymerase chain reaction (qPCR) test. In this study, tablet was chosen and three types of tablet formulation were selected; uncoated, film-coated and sustained-release tablets. Porcine DNA detection was assessed in each excipient used in formulating the three types of tablets. A template of positive and negative controls of uncoated tablet, film-coated tablet and sustained-release tablet were developed according to pharmaceutical standard for porcine DNA detection. Porcine and bovine gelatines were used as a spiking agent in each excipient and as a binder in positive and negative controls tablets. As a result, porcine DNA was detected in all spiked excipients and positive control tablets. It was found that CTAB solution was able to entrap DNA from polysaccharide matrix contained in sodium starch glycolate and pregelatinised starch. The results indicated that kaolin contained in coating excipients has a high affinity to DNA and in alkaline condition, the tight interaction of kaolin and DNA can be weakened. The results showed that porcine DNA was still able to be retrieved and detected after the drying process for uncoated tablet production. Thus, porcine DNA detection using qPCR testing method can be applied to detect porcine content in tablet and its excipients, when certain extraction techniques are adhered to.

CHAPTER 1 : INTRODUCTION

1.1 AWARENESS ON HALAL PRODUCT

Halal is a term that has its origins from the Arabic language. It is defined as things or actions that are permitted by the Shariah law (Law of Islam). Haram is the opposite word for Halal, and it refers to actions or things that are prohibited for Muslims. A halal product can be defined as a product that is free from haram sources such as pork or porcine derivatives and is produced in a hygienic manner. A halal product can also be generally defined as safe, hygienic and of high quality (Aziz et al., $2^0$12). In recent years, halal is not only being applied to food and beverage. These non-food halal products range from cosmetic, pharmaceutical, packaging to personal care. Beyond that, halal services are also emerging in recent years in the finance, insurance, tourism, entertainment, testing laboratory and logistic industries (Rahim et al., 2013). The awareness of non-Muslim consumer towards halal verification is increasing as halal products are known to be safer, of good quality and the animals are slaughtered in a less harmful way (Zainalabidin et al., 2010). In addition, the awareness of consumers towards halal product is not only limited to processed meat and food but they also start to seek halal pharmaceuticals and cosmetics, as shown in Figure 1.1 (Zain, 2011).

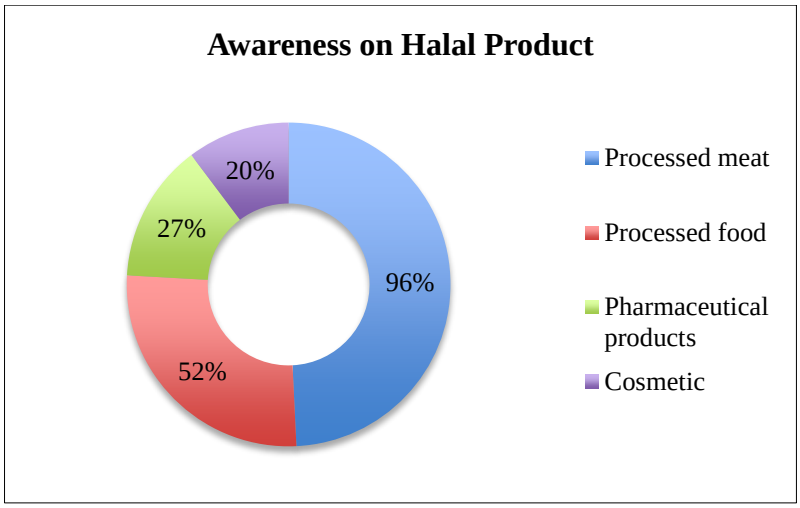

Figure 1.1 Percentage of awareness indicating the Malaysian consumers' demand of halal requirements towards different products (Zain, 2011)

1.2 MARKET DEMAND ON HALAL PHARMACEUTICAL PRODUCTS

Currently, market demands on halal pharmaceutical products are tremendously increasing because the Muslim population is the second largest religious community in the world as shown in Figure 1.2 (CIA, 2013), and halal adherence in products has become increasingly sought out as it is a Muslim's obligation to conform to the Holy Quran. According to Pew Research Centre (2009), Muslim population in the world will massively grow from 1.6 billion in 2010 to 2.8 billion by 2050. As the Muslim population will grow exponentially within the upcoming years, it is expected that there will a positive growth in the halal market business. In addition, global halal market was projected to be worth US$580 billion a year (Aziz et al., 2012) and halal pharmaceutical products constitute a fragment of this growing market.

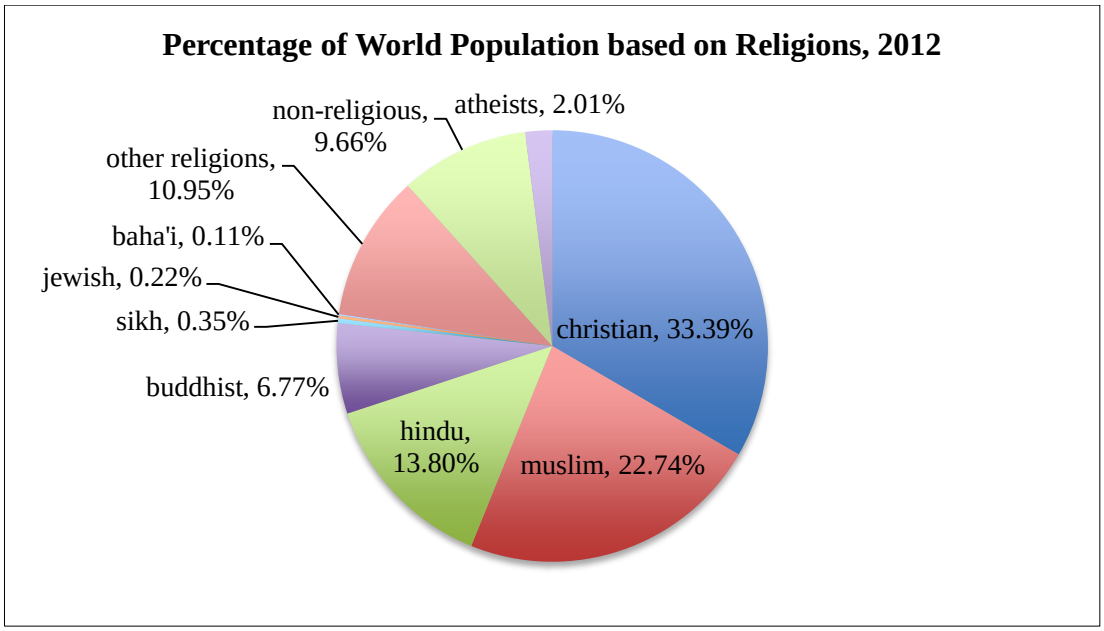

Figure 1.2 Percentage of world population based on religion. (CIA, 2013)

In April 2011, Malaysia has established its own halal pharmaceutical standards – Malaysian Standards MS2424:2012 and it is the first in the world (Peng et al., 2012). Halal pharmaceutical standards are defined as pharmaceutical products that comply with good manufacturing practices (GMPs) and halal quality assurance. The standard is developed as a guideline to all pharmaceutical industries intending to produce a halal pharmaceutical product that is safe to be consumed and is hygienic.

High market demand for halal pharmaceutical products and the establishment of halal pharmaceutical standards have led to the emergence of halal pharmaceutical and nutraceutical companies. Chemical Company of Malaysia Berhad (CCM) was the first pharmaceutical company in Malaysia that produced halal certified supplement products. Beside CCM, Safwa Health supplements also produced several halal certified supplement products that are manufactured in Australia. In Brunei, Simpor Pharma is the first pharmaceutical company to produced halal certified supplement products (Zaili, 2015).

1.3 CURRENT HALAL VERIFICATION METHOD ON HALAL PHARMACEUTICAL PRODUCTS

1.3.1 Consumer

Generally, a Muslim consumer verifies that a product, including health supplements, is halal by checking the authentic halal logo labelled on the packaging visually. Alternatively, a list of halal pharmaceutical products can be obtained from a local halal regulatory body portal such as Halal Malaysia Official Portal. Well-informed Muslim consumers will check the source of active ingredient and excipients contained in the product. In certain cases, they would ask directly from the pharmaceutical company when there are animal-derived excipients contained in the product but no halal logo on the packaging.

1.3.2 Halal regulatory body

A halal regulatory body is designated for halal certification and enforcement, and it verifies whether the processes and ingredients used comply with the Shariah law. Examples of halal regulatory bodies are the LPPOM MUI of Indonesia, Department of Islamic Development Malaysia (JAKIM), Islamic Food and Nutrition Council of America (IFANCA), Islamic Food and Nutrition Council of Canada (IFANCC) and Office of Muslim Affairs of the Philippines.

The verification methods for pharmaceutical products used by JAKIM are done on-site and paper audits. In certain situations, the regulatory body will send the product or evidence to halal testing laboratory when the product is suspected to be contaminated or adulterated with haram sources.

1.3.3 Researchers

To date, most published literatures regarding halal verification methodology are based on the presence of haram sources such as porcine and trace of alcohol. In recent years, researchers are focusing on porcine trace detection method in meat and processed food as well as alcohol level detection, due to the higher susceptibility of these products to be adulterated with porcine-derivative or high alcohol contents (Regenstein et al., 2003; Man et al., 2011; Senyuva et al., 2013). Halal verification on porcine trace detection in pharmaceutical products is still at its infancy.

1.4 DNA AS TARGET TOOL FOR PORCINE TRACE DETECTION

In the analytical method for porcine trace detection, a target or marker is needed for quantitative and qualitative analysis. Currently, there are various targets used for porcine trace detection such as protein, fatty acid, RNA and DNA. Protein analysis is efficient for detection in unprocessed products (Zhang et al., 2007), fatty acid analysis is effective mainly for animal-derived fats (Lumley et al., 1996). RNA analysis is difficult compared to other analysis because it requires an RNAse-free technique in addition to RNA being an unstable molecule (Tan et al., 2009).

DNA analysis is the most common target tool for porcine trace detection in processed products (Mafra et al., 2008) because DNA has proven to be able to withstand product processing better than other targets such as protein (Woolfe et al., 2004). Compared to protein, DNA carries much more of the organism's genetic information and it is a relatively stable molecule in the cell, allowing species identification in processed products (Hammes et al., 1995). The limitation of using DNA as a target, however, is the low quantity of DNA in processed product, and it can be damaged and degraded into small fragments due to exposure to harsh

condition such as mechanical shearing. Fortunately, DNA is amplifiable through the polymerase chain reaction (PCR) process.

1.4.1 DNA extraction and isolation method

In 1869, a Swiss physician, Friedrich Miescher was the first to perform DNA isolation from cells (Mischer, Miescher, 1869; Dahm et al., 2005). Current DNA extraction and isolation method has been further optimized to obtain a purified DNA in an effective way (Tan et al., 2009).

DNA extraction and isolation method is also known as nucleic acid extraction. There are two types of nucleic acid extraction, which are conventional method and solid-phase extraction. One of the examples for conventional method is cetyltrimethylammonium bromide (CTAB) extraction method. This method is usually applied to extract DNA from plant sample, in which CTAB, a cationic surfactant will precipitate the polysaccharide matrix contained in the plant extract (Sambrook et al., 2001). Commercial DNA purification kits available in the market commonly applied solid-phase nucleic acid extraction because this extraction method is efficient in purification of DNA, and it is less time-consuming compared to the conventional method (Esser et al., 2005). The extraction is efficient because less amount of sample is needed and the application of rapid centrifugation and column separation during the extraction steps make the duration of nucleic acid purification process faster (Gjerse et al., 2009). Silica matrices, glass particles, and magnetic bead-based nucleic acid purification are several examples of solid-phase nucleic acid extraction. Silica matrix is the most basic matrix used for nucleic acid purification.

Generally, a combination of conventional method and solid phase extraction is necessary to maximise the amount of extracted DNA from a sample that contains matrices or contaminants especially samples from processed products. When contaminants or matrices are present in extracted DNA, it will become an inhibitor and disrupt the process of DNA quantification and detection. Proteinase K, an enzyme that digests protein also plays an important role during DNA extraction in processed product that is heterogeneous. This enzyme cleaves the peptide bond in proteins and works optimally at 65°C. A purified DNA sample will indicate that the DNA extraction and isolation method has been carried out efficiently.

1.4.2 Quantitative polymerase chain reaction (qPCR) testing method for porcine DNA detection

In 1983, Kary Bank Mullis together with Michael Smith invented the PCR technique, and they were later awarded with a Nobel prize (Mullis et al., 1987). PCR works by enzymatically replicating a small amount of extracted DNA and amplifying it repeatedly in several cycles to produce multiple copies of DNA (Saiki et al., 1988).

The first instrument used for DNA amplification was a thermal cycler, which became commercially available in 1987. In recent years, quantitative PCR (qPCR) is one of the latest instruments used for DNA detection. qPCR testing is a sensitive and effective method to detect specific DNA fragment which is available only in a small quantity. Hence, qPCR is commonly used for DNA detection in forensic samples and GMO products (Alonso et al., 2004). In porcine DNA detection, qPCR is also commonly used to identify porcine trace in meat, processed food and gelatine. qPCR can monitor the output of DNA amplification with fluorescence marker (McCartney

et al., 2003). At certain PCR cycle, the fluorescence signal will start to emit, showing the presence of targeted DNA. Cycle threshold (Ct) value is used to measure the amplified DNA. A low Ct value would indicate a high amount of DNA template present in the extracted DNA sample.

As mentioned earlier, qPCR testing method is based on the detection of fluorescence signal emitted during PCR cycle. The source of fluorescence signal can be divided into two categories. The first category applies DNA-intercalating agents such as SYBRGreen I and EvaGreen (Navarro, 2014). DNA-intercalating agent is the simplest signal agent, but it detects both specific and non-specific DNA target. On the other hand, the second category uses fluorescence probe attached to oligonucleotides such as TaqMan probe. This hydrolysis probe consists of reporter fluorophore at 5' end and quencher at 3' end, and it only detects specific amplicon (Figure 1.3).

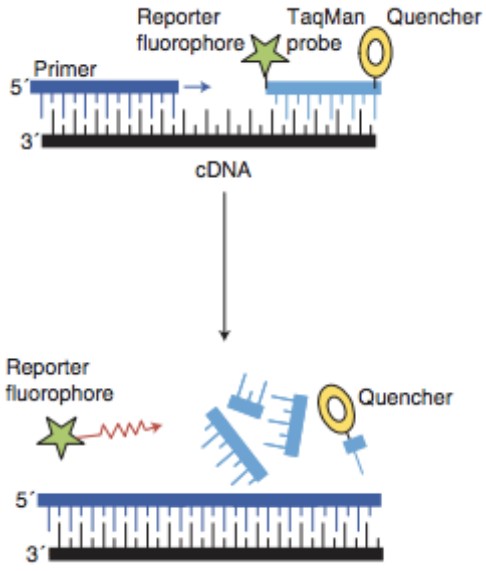

Figure 1.3 TaqMan probe (Arya et al., 2005)

The selection of a specific DNA target template plays a key role in the specificity of qPCR testing method for porcine DNA detection. Mitochondria DNA (mtDNA) was commonly used for DNA target template in animal species identification (Zhang, 2006; Man et al., 2010; Ali et al., 2012;). The advantage of mtDNA is that it is abundant in mitochondrion (Wiesner et al., 1992) compared to DNA which is contained in cell nucleus or nuclear DNA. Besides, there are in range of 1000 to 2000 mitochondria that can be found in each cell (Radu et al., 2009). These will help in retrieval of DNA from highly processed products. MtDNA is highly evolved and diverse compared to nuclear DNA, thus DNA similarity between species that are closely related can be reduced (Wolf et al., 1999).

PCR inhibitor can be defined as any foreign substance contained in extracted DNA sample that will disrupt PCR sensitivity by interfering with PCR analysis, leading to false negative results being obtained. The inhibitors are commonly found in food, processed product and environmental samples (Table 1.1). Besides, applying a poor DNA extraction and isolation technique and using contaminated reagent may also contribute to the presence of PCR inhibitor during PCR analysis. This problem can be countered by identifying the potential PCR inhibitor present in analysed sample, performing DNA extraction and isolation in a clean environment and washing out PCR inhibitor by optimizing DNA extraction and isolation method.

Table 1.1 List of common PCR inhibitors (Bessetti, 2007; Shrader et al., 2012)

Inhibitor	Source of Inhibitor
Complex polysaccharides	Plant material, stool
Collagen	Tissues
Proteinases	Milk
Indigo dye	Denim
Surfactant	Buffer
Clay and its derivative	Soil

In commercial DNA detection kits, internal PCR control (IPC) or external DNA target control is used to identify the presence of PCR inhibitor during DNA amplification. This control is commonly made of synthetic oligonucleotide that has no similarity with target sequence and is amplified together with the DNA samples (Kontanis et al., 2006).

1.5 TABLET AS A PHARMACEUTICAL DOSAGE FORM

Tablet is the most common type of pharmaceutical dosage form and is frequently found in the market especially as prescribed medicines and health supplement products. The advantages of using tablet as an oral dosage form are that it is a convenient form of drug administration, it can be produced in uniform dose from tablet to tablet on high-speed compression, and it is also stable in extended and diverse storage conditions (Lieberman et al., 1989; Aulton, 2007).

There are various types of tablet formulations; they include compressed tablet, prolonged release tablet, coated tablet, disintegrating tablet, chewable tablet,

effervescent tablet and lozenges (Lieberman et al., 1990). The most common compressed tablet formulation is the uncoated tablet. Uncoated tablet can be produced by direct compressing, dry granulation and wet granulation. Direct compression is applied using tablet press instruments such as single-punch press, rotary press and computerized hydraulic press (Aulton, 2007). Dry and wet granulation methods are similar except dry granulation is favourable for active ingredient that is sensitive to heat and moisture.

The formulation of uncoated tablet was further improved to coated tablet formulation especially to protect the drug from light and moisture. In coated tablet formulation, there are three main types that are commonly used in the pharmaceutical industry; film coating, sugar coating and compression coating (Porter, 1999). In film coating, polymer is sprayed to cover the whole tablet surface and form a thin film. This form can be produced at high speed and therefore it is favourable compared to other coated tablet formulation.

In recent years, modified release mechanism is desirable due to many advantages compared to immediate release mechanism. Modified release mechanism involves a continuous drug release in the body to sufficiently provide a prolonged duration of therapeutic action. The mechanism can be of benefit by reducing the drug dosage per day, which can lead to cost savings and less gastrointestinal side effects (Chien, 1995). There are various terms that fall under this modified release definition; they include delayed-release system, sustained-release system, extended-release system and controlled-release system. Sustained-release tablet formulation made up of a

hydrophilic matrix delivery system is less complexed to be produced at lab scale because the preparation method is similar to wet granulation method.

1.5.1 Sources of non-halal excipients that are potentially found in tablets

A tablet consists of active ingredients and several excipients used to complete a tablet formulation and ease the tableting operation. An excipient is a processed product and can be categorized based on its function – diluent/filler, disintegrant, binder, lubricant and glidant. Diluent/filler is commonly used in tablet formulations when the amount of active ingredient is low and the filler is needed to be added to increase the tablet weight to at least 50 mg. Lactose is the most common filler besides cellulose and sucrose because it is chemically inert, non-hygroscopic and has a tolerable taste. Disintegrant also plays an important role in tablet formulation. It promotes the tablet to disintegrate after consumption. Starch made from potato, maize and corn was the traditional disintegrant used in tablet formulation. Currently, modified starches such as pregelatinised starch and sodium starch glycolate are typically used and are effective disintegrants. A binder or adhesive is used to bind excipients mixture with drug during the granulation process. Gelatine and starch were the common traditional binders. Nowadays, polymers such as polyvinylpyrrolidone (PVP) and cellulose derivatives are used instead because of better adhesive properties. Glidant is used to improve the flowability of granules during tableting press process. The most commonly used glidant is colloidal silica because the particle of silica is very small and can adhere to the other ingredient surfaces. During tablet press, lubricant act to reduce the friction between the granules and the die wall. The most effective lubricants are made from stearic acid and stearic acid salts. Magnesium stearate is widely used as a lubricant in tablet formulation. As

shown in Table 1.2, there is number of excipients that are derived from animal sources.

Table 1.2 List of common excipients derived from animal sources (The Halal Index, 2011)

Excipients	Sources	Uses
Gelatine	Porcine & bovine	Binder
Magnesium stearate	Porcine, bovine & plant	Lubricant
Stearic acid/ stearates	Porcine, bovine & plant	Lubricant
Lactose monohydrate	Porcine & bovine	Filler
Glycerine/ Glycerol	Porcine, bovine & plant	Humectant sweetening agent

There is a potential that a tablet or its excipients may be adulterated or contaminated with haram sources such as gelatine from porcine source. Certain pharmaceutical companies did not declare the source origin of their excipients especially gelatine (Hussain, 2010). Most gelatine produced in United Kingdom (UK) was 80% made from porcine source because porcine is readily available compared to bovine gelatine (Boran et al., 2010). In 1986, UK reported 180,000 cases of Bovine Spongiform Encephalopathy (BSE) or widely known as mad cow disease, which is a neurodegenerative illness that can be deadly (Harman et al., 2009). Due to the disease outbreak, pharmaceutical companies in the UK started to replace their gelatine source from bovine to porcine. Unfortunately, these changes posed a threat to Muslims and Jewish consumers as consumption of pork or any of its derivative is prohibited by the religious laws of Islam and Judaism (Ali et al., 2012).

1.6 PROBLEM STATEMENT AND OBJECTIVES OF STUDY

Awareness on halal pharmaceutical products have increased in recent years. This phenomenon is led by Muslim population that is widespread around the world and it also benefits other religion and belief systems such as Jewish and vegan communities. Currently, there is a need for analysis of porcine trace or adulteration in halal pharmaceutical products due to religious belief and also due to the use of undeclared animal species in halal products. In Malaysia Halal Pharmaceutical Standard (MS 2424:2012), there is no definite method to determine animal DNA contamination, specifically porcine DNA, in heterogeneous pharmaceutical products during tableting process and in excipients. Thus, a proper study on detecting porcine contamination at molecular level is essential to address this issue.

During tableting process, porcine DNA shearing may occur. Also, porcine DNA may be entrapped by an excipient that has a high affinity to DNA. These factors will give an inaccurate result on porcine DNA detection. The aim of this study is to identify factors within tablet formulation and its processing that could hinder the detection of porcine DNA and subsequently develop a method that could circumvent these obstacles.

The objectives of this study encompass the following:

- To formulate and prepare a template of adulterated tablet with porcine gelatine which conform to pharmaceutical standards.
- To determine the crucial steps in tableting process that may affect porcine DNA recovery.

- To investigate the effect of excipient on porcine DNA detectability by qPCR testing method.
- To develop an optimized DNA extraction and isolation method on tablet components that may hinder porcine DNA detection.

CHAPTER 2 : PORCINE DNA DETECTION IN UNCOATED TABLETS AND THEIR EXCIPIENTS

2.1 INTRODUCTION

Generally, tablets can be produced by dry or wet granulation methods depending on the active ingredient properties (Chaudari et al., 2012). In this study, wet granulation was chosen to produce uncoated tablets because this method is commonly used in pharmaceutical industry (Agrawal, 2011). Excipients were selected to suit wet granulation method, which contained filler, disintegrant, binder and lubricant as basic components in uncoated tablet formulation. In addition, gelatine was selected as a binder agent, the material from porcine source could be formulated as the positive control uncoated tablet. The selected excipients were chosen based on those commonly used in wet granulation for immediate release uncoated tablet and mostly from animal or plant-derived.

The main step in uncoated tablet preparation are mixing, granulation, drying and compaction. The extra step in wet granulation that differs from dry granulation is the drying step. There is a possibility that DNA recovery will be affected during this step due to drying the wet granules at high temperature (Hupfer et al., 1998).

The aims of this chapter comprise the following:

- To determine which excipient(s) used in formulating uncoated tablet could hinder porcine DNA recovery.
- To perform porcine DNA detection on a template of adulterated uncoated tablet with porcine gelatine.

- To investigate the effect of drying the tableting process on porcine DNA recovery.

2.2 MATERIALS AND METHODS

The excipients used were lactose monohydrate (DFE Pharma, Germany), microcrystalline cellulose (Avicel PH-102, Mingtai Chemical, Taiwan), sodium starch glycolate (Yung Zip Ltd, Taiwan), magnesium stearate (RMK Chemical, India), bovine gelatine bloom 180 (Leverage, Malaysia). Porcine gelatine bloom 180 (Sigma-Aldrich, Germany) was used as a spike material.

2.2.1 Selection of excipients for uncoated tablet formulation

The sources of origin for all excipients were mostly from plant source. The excipients used in formulating immediate release uncoated tablets are listed in Table 2.1.

Table 2.1 Excipients used in the formulation of immediate release tablet

Excipients	Functional category	Source
Lactose monohydrate	Filler/diluent	Bovine
Gelatine	Binder	Bovine & porcine
Microcrystalline cellulose (MCC)	Disintegrant	Plant
Sodium starch glycolate	Disintegrant	Plant
Magnesium stearate	Lubricant	Plant & animal

2.2.2 Porcine DNA detection on excipients used in the formulation of immediate release uncoated tablets

In this step, problematic excipient(s) that may hinder porcine DNA recovery was determined. Porcine DNA detection was carried out on unspiked and spiked excipients with porcine gelatine.

2.2.2(a) Sample preparation

The four selected excipients excluding gelatine (Table 2.1) used for formulating immediate release uncoated tablet were contained in two excipients groups. The first group of excipients was unspiked and the second group was spiked with porcine gelatine.

Each excipient for group 1 was weighed approximately 100 mg and transferred into a labelled 1.5 ml sterile microcentrifuge tube. Excipient for Group 2 was weighed separately for 1 g and mixed with 1 ml of 8% w/v porcine gelatine solution in a clean mortar and pestle. Then, 100 mg of wet mixture of Group 2 was weighed and transferred into a labelled 1.5 ml sterile microcentrifuge tube. All samples including porcine gelatine as internal positive control and bovine gelatine as negative control were prepared in duplicate for DNA extraction and isolation. The summary of sample preparation on excipients was shown in Figure 2.1.

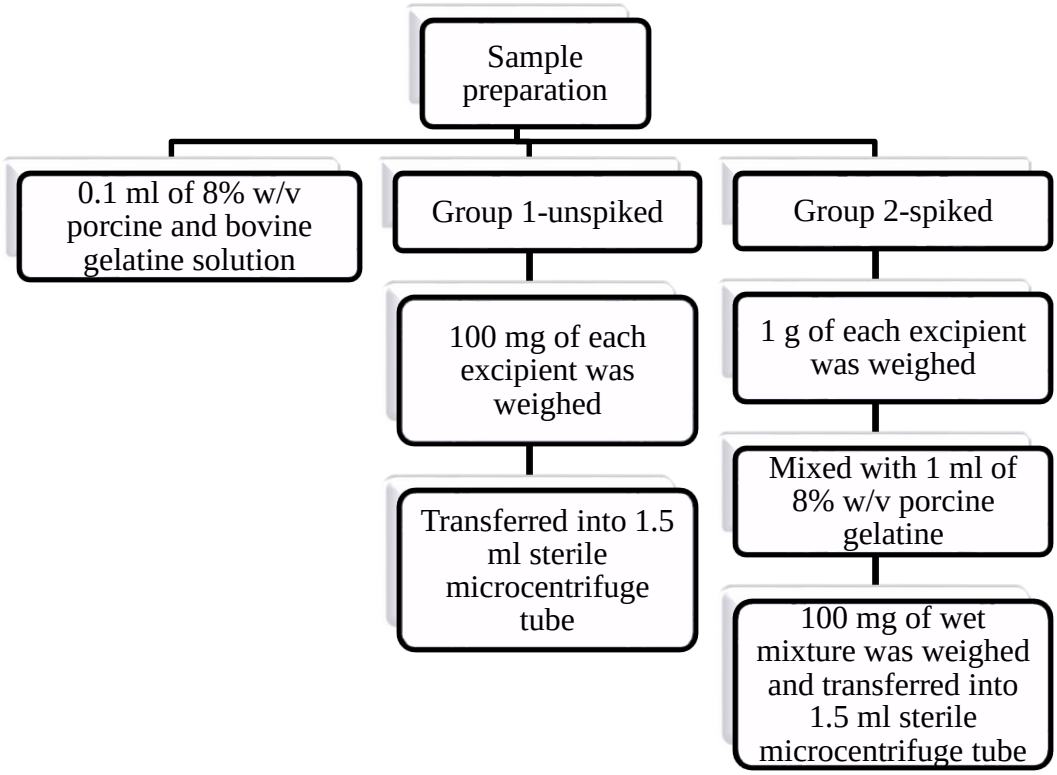

Figure 2.1 Flow chart of summary of sample preparation on excipients

2.2.2(b) DNA extraction and isolation

The procedure of DNA extraction and isolation for lactose monohydrate, MCC, gelatine and magnesium stearate was carried out as suggested in Agilent DNA Isolation kit (Agilent Technologies, USA) protocol, which contains nucleic acid binding buffer, high salt wash buffer, elution buffer, DNA-binding spin cups and 2 ml receptacle tubes, 1.5 ml collection tubes, Proteinase K and Proteinase K digestion buffer. Sodium starch glycolate used different DNA extraction and isolation kit from other excipients, which is DNeasy *Mericon* Food kit (Qiagen, Germany) that contains Food Lysis buffer, Proteinase K, QIAquick® spin column, PB buffer, AW2 buffer and EB buffer. DNA extraction and isolation on sodium starch glycolate was conducted according to DNeasy *Mericon* Food kit protocol.

The DNA extraction and isolation started when 20 µl of Proteinase K and 200 µl of Proteinase K Digestion buffer were added and mixed into all samples tubes. Then, the tubes were incubated at 65°C with constant shaking at 800 rpm for 1 hour in a thermomixer (Thermomixer Comfort, Eppendorf, Germany).

After 1 hour incubation, all tubes were centrifuged in a centrifuge (5424, Eppendorf, Germany) at 11,500 rpm for 6 minutes. Next, 150 µl of supernatant was collected from each tube and transferred into a new 1.5 ml sterile microcentrifuge tube containing 500 µl of nucleic acid binding buffer. The tube was vortexed using a vortex mixer (Vortex Genius 3, IKA, Germany) for 10 seconds to ensure a homogenous distribution mixture.

Then, the mixture was transferred into DNA binding spin cup column placed in a 1.5 ml collection tube. The collection tube was centrifuged at 14,680 rpm for 1 minute.

After centrifuged, the flow-through contaminant contained in the collection tube was discarded. Next, 500 µl of 1X high salt wash buffer was added into each DNA binding spin cup column and centrifuged at 11,100 rpm for 1 minute. The flow-through contaminant contained in the collection tube after centrifuged was discarded and 500 µl of 80% v/v ethanol was added to wash away any salt residual on the column. Next, the collection tube was centrifuged at 11,100 rpm for 1 minute and the flow-through of the ethanol contained in the collection tube was discarded after centrifuged. This washing step of salt residual was repeated twice. Then, the collection tube was centrifuged again at 14,680 rpm for 2 minutes to dry the silica column membrane from ethanol residual.

The DNA binding spin cup column was then placed in a new 1.5 ml collection tube and 100 µl of elution buffer taken that was being pre-heated from the thermomixer was added directly onto the DNA binding spin column membrane surface. After incubating DNA binding spin cup column at room temperature for 1 minute, the column was centrifuged at 14,680 rpm for 1 minute to elute the DNA adsorbed on DNA binding spin cup column membrane. Next, the DNA binding spin cup column was discarded. The eluted DNA collected in the collection tube was further tested for DNA purity and concentration.

2.2.2(c) Assessment of DNA purity and concentration

The quality and concentration of extracted DNA was determined using NanoVue Plus UV Vis spectrophotometer (GE Healthcare, UK). Elution buffer was used as a reference sample. A volume of 1 µl DNA sample from each excipient was placed onto the horizontal hydrophobic surface plate. The DNA concentration and purity of each excipient was measured and the results shown from spectrophotometer were

recorded. DNA concentration was calculated by multiplying OD_{260} reading with 50. DNA purity was determined by calculating the ratio of OD_{260} reading/OD_{280} reading. The DNA sample with purity value range of 1.8 to 2.0 was used for porcine DNA detection procedure.

2.2.2(d) Porcine DNA detection

The amplification and detection of porcine DNA was performed using a commercial kit, Agilent Porcine Detection QPCR kit (Agilent Technologies, USA). All extracted DNA and controls, which are no-template control (NTC) and porcine DNA control supplied from the commercial kit, were prepared in triplicate. Porcine DNA control acts as a positive control in qPCR reaction. The porcine detection assay mix supplied from the kit, contains PCR primers, fluorogenic probe and external DNA control template or "Alien DNA". PCR reagent mixture was prepared in a 1.5 ml sterile microcentrifuge tube, which consists of 12.5 µl qPCR master mix and 2.5 µl porcine detection assay mix. The final volume of PCR reagent mixture was prepared in a 1.5 ml sterile microcentrifuge tube. The volume for each components added in each reaction tube are as shown in Table 2.2.

Table 2.2 Volume of components added in each reaction tube

Components \ Reaction Tubes	NTC	Excipients	Positive control
PCR reagent mixture	15 µl	15 µl	15 µl
Porcine DNA control	-	-	10 µl
Nuclease-free water	10 µl	-	-
DNA extract	-	10 µl	-
Final amount	25 µl	25 µl	25 µl

Subsequently, all reaction tubes were briefly spun in a mini centrifuge. The reaction tubes were placed in the thermal cycler, Mx3005P QPCR systems (Agilent Technologies, USA). Then, FAM and Cy5 channels were selected from the thermal cycler's software. Next, the qPCR thermoprofile programme was run as shown in Table 2.3 and start to run the porcine DNA amplification. The starting point for fluorescence signal detection was during annealing and elongation stage. After the run had completed, the data analysis was recorded.

Table 2.3 qPCR thermal profile programme

PCR stage	Number of Cycles	Temperature (^{0}C)	Duration
Initial	1	95	10 minutes
Melting point	40	95	30 seconds
Annealing & elongation		60	1 minute

2.2.2(e) DNA extraction and isolation of sodium starch glycolate using CTAB method

DNA extraction and isolation began when 1 ml of Food Lysis buffer contained cetyltrimethylammonium bromide (CTAB) and 2.5 µl of Proteinase K were added and mixed into sample tubes. Then, the tube was incubated at 60°C with constant shaking at 1000 rpm for 30 minutes in a thermomixer. After the incubation, the tube was cool down for 15 minutes in a cold block. Then, the tube was centrifuged for 5 minutes at 5,150 rpm. After centrifugation, 700 µl of supernatant was withdrawn from each tube and transferred into a new 1.5 ml sterile microcentrifuge tube containing 500 µl of chloroform. The tube was vortexed for 15 seconds and centrifuged at 12,200 rpm for 15 minutes.

Next, 350 µl of supernatant from each tube was collected after centrifugation completed. The supernatant was transferred into a new 1.5 ml sterile microcentrifuge tube containing 350 µl of PB buffer. The tube was vortexed for 15 seconds to ensure a homogenous mixture. Then, the mixture was transferred into a QIAquick spin column placed in a 2 ml collection tube. The collection tube was centrifuged for 1 minute at 13,800 rpm. After centrifugation, the flow-through solution in the collection tube was discarded. Next, 500 µl of AW2 buffer was added into the QIAquick spin column and recentrifuged at 13,800 rpm for 1 minute. The flow-through solution contained in the collection tube was discarded and the spin column was recentrifuged again at 13,150 rpm for 1 minute to dry the membrane.

The QIAquick spin column was transferred into a new 1.5 ml sterile microcentrifuge tube and 150 µl of EB buffer was added directly onto the QIAquick spin column membrane surface. After incubating the QIAquick spin column at room temperature for 1 minute, the column was centrifuged at 13,800 rpm for 1 minute to elute the DNA adsorb on QIAquick spin column membrane. Next, QIAquick spin column was discarded and the tube containing eluted DNA was further tested for DNA purity and concentration assessment as mentioned in section 2.2.2(c) and proceed with porcine DNA detection as mentioned in section 2.2.2(d).

2.2.3 Preparation of uncoated tablets

In this study, a placebo uncoated tablet was formulated with selected excipients from those commonly used in wet granulation. The formulation for uncoated tablet was referred from a formulation that utilizes povidone (PVP) as a tablet binder. The original formulation was modified by replacing PVP to gelatine and suitable concentration of gelatine solution was identified. In order to optimise

the formulation, two concentrations of gelatine solutions were used in this formulation, which are 10% w/w and 8% w/w of gelatine solution. The formulations were prepared with excipients as listed in Table 2.4.

Table 2.4 Formulations of uncoated tablet

Excipients	Formulations	
	F1	F2
Lactose monohydrate	392.0	401.8
Gelatine	49.0	39.2
MCC	14.7	14.7
Sodium starch glycolate	29.4	29.4
Magnesium stearate	4.9	4.9
Total tablet weight (mg)	490	490

First, 10% w/w of gelatine solution from bovine source was prepared by dissolving in distilled water at 45°C. All excipients were weighed and except magnesium stearate were transferred and mixed in a plastic bag for 5 minutes. Then, pre-blend was transferred into a planetary mixer (KM010 mixer, Kenwood, UK) and mixed at speed of 10 rpm for 2 minutes. Next, half volume of 10% w/w gelatine solution was added and mixed at speed of 40 rpm for 5 minutes. The end point of wet mixing was achieved when a smooth crack surface appeared by pinching the wet powder blend at 90°. Later, the wet powder blend was granulated using granulator (YK 60B, China Pharmaceutical Machinery Factory, China) with sieve size 800 μm, followed by drying of the wet granules in fluidized bed dryer (TG 200, Retsch, Germany) for 8 minutes at 65°C. Then, the dried granules were transferred into the planetary mixer and wet mixing was repeated using another half of gelatine solution. Next, the wet mixing was re-granuled using granulator and dried again using fluidized bed dryer for 8 minutes at 60°C. Finally, the dried granules were screened from dried powder blend using automated sieve (AR 401, Erweka, Germany) with sieve size 630 μm