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FOODBORNE PATHOGENS ON MEAT STORED IN MAJOR CENTRAL COLD ROOMS IN IBADAN AND THEIR SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

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ABSTRACT

Foodborne pathogens are the leading cause of illness and death in developing countries and are often associated with poor hygiene and unsafe food storage conditions. Using central cold rooms with alternate power supply in preserving meats due to erratic power supply is common among meat traders in Nigeria. However, the public health safety of the operations of this practice remains un-investigated. We conducted a microbial assessment of aseptically collected meat swabs from three selected major cold rooms in Ibadan for *Staphylococcus aureus, Listeria monocytogenes, Salmonella* spp. and *Escherichia coli* using standard procedures. Antibiotic susceptibility was determined using 14 different antibiotics at standard concentrations following Kirby-Bauer Assays. The data were analysed with Stata 12.0 using bivariate and logistic regression analyses. Of 180 meat swabs collected, 42.2 % were positive for *S. aureus*, 22.2 % for *L. monocytogenes*, 20.0 % for *Salmonella* spp. and 6.7 % for *E. coli*. All of the isolates exhibited total resistance to seven of the antibiotics. *Escherichia coli* showed the highest resistance to 12 antibiotics, followed by *Salmonella* spp. (11 antibiotics), *L. monocytogenes* (10 antibiotics) and *S. aureus* (7 antibiotics). Sampling locations were significantly associated with the prevalence of *L. monocytogenes* (P = 0.008) and *S. aureus* (P = 0.000), but not with *Salmonella* spp. (P = 0.435) or *E. coli* (P = 0.117). The study revealed a heavy microbial contamination with major foodborne pathogens characterized by a high level of antibiotic resistance. These findings portend that the current operations associated with the practice of using central cold rooms in meat preservation in Nigeria undermine public health safety and need to be urgently addressed.

Key words: antimicrobial resistance; cold storage; food safety; meat; microbial contamination; public health

INTRODUCTION

Like most developing countries of the world, Nigeria is facing a food crisis. The diet of most of her populace is

often protein deficient, with the average animal protein consumption per person per day being 7.9 g which is far below the 35 g recommended by the Food and Agriculture Organisation [21, 32]. This low level of animal protein intake has generated concern as it affects the physical, physiological and mental development of Nigerian youths and the labour force in the country [5]. There is, therefore, the need for conscious efforts towards adequate availability of animal products that are rich in protein, energy, vitamins and minerals required for adequate nutrition. One major animal product that has received attention over the years is meat. Though highly nutritious because of its high biological value protein [8], meat provides a veritable medium for the growth and multiplication of micro-organisms, making it highly perishable, poisonous and unfit for human consumption when contaminated with microbes [34]. This becomes more evident considering the inadequate refrigeration facilities for optimal preservation of meat coupled with erratic power supply that characterizes most developing countries, particularly Nigeria.

Foodborne pathogens pose a significant threat to public health, causing a substantial economic burden both in developed and developing countries [27, 42]. Recent epidemiological studies of world populations show that 420,000 people lose their lives every year due to foodborne diseases, with around one-third of those being five years of age or younger [24]. The recent outbreak of listeriosis in South Africa was the largest ever recorded involving 674 patients as of March 2018, with 27 % mortalities [45]. The identified Listeria strain (*L. monocytogenes* ST6) was traced back to the processing environment of a manufacturer of a ready-to-eat processed meat product (polony). As a result, 12 of the 15 African countries to which South Africa exports, recalled and banned imports of the affected meat products, while the rest banned imports of all food products [45]. In Nigeria, there have been several reports of cases of illnesses and deaths due to foodborne pathogens [6, 37, 39], some of which were linked to contaminated meat. Most of the pathogens responsible for diarrhoea in Nigeria are zoonotic, with cattle as an important reservoir for many. Beef consumption has been associated with a nine-fold increase in the odds of experiencing diarrhoeal illness with additional four-fold increase in the odds when consuming beef of poor microbiological quality. The cost associated with meat-borne diarrhoea alone in Nigeria has been estimated at US$ 156 million [30]. However, proper handling and storage of meat limits the bacterial contamination or multiplication, thus reducing the associated cost due to meat-borne diseases.

The use of central cold rooms, a temperature-controlled facility designed to preserve meats for the teeming population is a common practice among meat traders in developing countries such as Nigeria. This practice is necessitated by irregular power supply which characterizes such countries given the alternative power supply to the facility. However, the public health safety of the operations of this practice in terms of optimal preservation of meats from microbial pathogens is yet to be seen. The aim of this study was to carry out microbial assessment of meats stored in major cold rooms in Ibadan, south-western Nigeria for *Escherichia coli, Listeria monocytogenes, Salmonella* spp., and *Staphylococcus aureus*, the four commonly reported pathogens of public health importance as well as to determine their susceptibility to antimicrobial agents.

MATERIALS AND METHODS

Study site

This cross-sectional study was carried out in Ibadan, south-western Nigeria between August and November, 2017. Ibadan is the largest city in terms of the geographical area in the country and one of the country's most populous cities with over three million people. The city accommodates a major central abattoir which supplies meat to the teeming population in the city as well as neighbouring communities in the south-western region. Meats are both sold at formal and informal markets mostly on a retail basis. Considering the challenge of storing meats until the next day, the meat traders in the region adopted the practice of storing their meats in central cold rooms powered by alternative power supplies. The study sites were the cold rooms present at Idi Ose and Ojoo areas of Akinyele Local Government Area (LGA), and Oniyanrin area of Ibadan North LGA. These cold rooms were of similar characteristics in terms of capacity, temperature, relative humidity and air circulation. They were small-sized cold rooms of about 1,000 m^3 capacity with temperature ranges between 0 and –20 °C and relative humidity of between 85–90 % with adequate airflow and uniformity.

Study population and sampling technique

The study population comprised all of the central cold rooms in the Ibadan metropolis. The cold rooms at Akinyele and Ibadan North LGAs were selected considering the presence of the state's abattoir and formal major meat markets in these areas. In addition, these cold rooms represented the major meat storage facilities for public meat traders in the Ibadan metropolis. The traders often stored and preserved their meat at the facilities to prevent meat spoilage and the associated decline in quality. The purpose of this study was explained to the managers of the cold rooms in order to seek their consent to swab samples of the meat stored in the cold rooms. Stored beef meats were aseptically swabbed on both dorsal and ventral parts by rolling sterile swabs against about 5 cm^2 area of the sampled items during unscheduled visits to the meat storage facilities in order to ensure the real situational assessment regarding the facilities. The samples were taken from the meat which had been stored at the cold room facilities not less than 18 hours. At least two samples were collected per sub-unit of the cold room facilities during each unscheduled visit. The samples were thereafter placed in the transport medium in the respective swab stick containers kept within cold chain and transported to the Food and Meat Hygiene Laboratory, Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, where sample processing took place. Peptone water and Listeria enrichment broth already prepared for the isolation of the respective organisms served as both transport media and selective broth for the incubation of associated organisms. The samples were processed within three hours of collection.

Sample processing

The swab samples in the broth media were incubated at 37 °C for 18 to 24 hours and then plated on the four primary agar media (Oxoid Limited, United Kingdom): Mannitol salt agar for *Staphylococcus aureus*, *Listeria* selective agar for *Listeria monocytogenes, Salmonella-Shigella* agar for *Salmonella* and Eosin-Methylene blue agar for *Escherichia coli*. After inoculation onto the agar, the Petri dishes were turned upside down and incubated at 37 °C for another 18 to 24 hours, (24 to 48 hours for *Listeria* plates only because of its relatively slow growth). After the incubation, the growths on the different media were observed. The most distinct colonies showing perfect morphological characteristics of the desired organisms on each medium were harvested and aseptically sub-cultured and incubated to obtain pure colonies.

Staphylococcus aureus were identified following the use of conventional biochemical methods including Gram staining, catalase, coagulase, sugar fermentation (mannitol, glucose, xylose and arabinose), haemolysis on 5 % sheep blood agar and pigmentation as described by Barrow and Feltham [10]. *Listeria monocytogenes* were identified as previously described [10] by employing the use of conventional biochemical methods including Gram staining, catalase, aesculin, triple sugar iron (TSI) reaction, motility, urease, sugar fermentation tests (lactose, sucrose, mannitol and xylose), motility and CAMP tests. Similarly, *Salmonella* isolates were identified by triple-sugar-iron (TSI) agar test and the urease test with a susceptible strain used as a positive control. *E. coli* was identified following the conventional biochemical tests, including urease production, catalase test, motility, Voges-Proskauer, indole production, carbohydrate fermentation tests, methyl red and citrate utilization.

Antibiotic Sensitivity Test (Kirby-Bauer Assay)

Antibiotic susceptibility testing was carried out using the Kirby-Bauer disc-diffusion test which was in line with the recommended standard as earlier described [16]. Briefly, an inoculum of each pure bacterial isolate was emulsified using 3 mL of sterile normal saline and the density thereafter was adjusted to the 0.5 McFarland standard. Inoculations into the Mueller-Hilton Agar (MHA) plates (Oxoid, England) were then carried out by a sterile cotton swab dipped into the standardized suspension of bacterial cultures. The plates were then allowed to dry. Antibiotic discs containing ciprofloxacin (5 µg), ofloxacin (5 µg), augmentin (30 µg), nitrofurantoin (300 µg), ampicillin (10 µg), ceftazidime (30 µg), cefuroxime (30 µg), gentamicin (10 µg), cotrimoxazole (25 µg), chloramphenicol (10 µg), cloxacillin (5 µg), erythromycin (5 µg), streptomycin (10 µg), tetracycline (10 µg) (Antibiotic Becton Dickinson and Company, Sparks, USA) were placed onto MHA plates. The plates were then incubated at 37 °C for 24 hours. Finally, the zone of inhibition was measured in millimetres and zone diameters interpreted in accordance with standards as susceptible, intermediate and resistant on the basis of the critical points recommended [16]. *S. aureus* ATCC 25923, *L. monocytogenes* ATCC7644, *En-*

terococcus faecalis ATCC 29212 and *Escherichia coli* ATCC 25922 were used as reference strains.

Data Analysis

The data were analysed using STATA 12. The frequencies and percentages were calculated as appropriate for the prevalence of each foodborne pathogen. Univariate binary logistic analysis and multivariate logistic regression analysis were conducted to determine the relationships between outcome variable (prevalence of the different foodborne pathogens) and sampling locations. Odds ratios (OR) were computed in order to determine the presence and strength of the associations between variables and 95 % Confidence Intervals (CIs) were calculated to investigate the statistical significance. Values of $P < 0.05$ were considered significant.

RESULTS

Prevalence of *S. aureus*, *L. monocytogenes*, *Salmonella* spp. and *E. coli* in stored meat

A total of 180 meat swab samples were collected from the three cold rooms, with 60 each from the three locations. Of the foodborne pathogens obtained, 42.2 % were identified as *S. aureus*, 22.2 % as *L. monocytogenes,* 20.0 % as *Salmonella* spp. and 6.7 % as *E. coli* (Table 1). Of the samples from the cold room at Idi Ose in Akinyele LGA, 52 (86.7 %) were positive for *S. aureus*, 4 (6.7 %) for *L. monocytogenes*, 16 (26.7 %) for *Salmonella* spp. and 8 (13.3 %) for *E. coli*. From Ojoo in Akinyele LGA, 4 (6.7 %) were positive for *S. aureus*, 24 (40.0 %) for *L. monocytogenes*, 12 (20.0 %) for *Salmonella* spp. and none (0.0 %) for *E. coli*. Again, out of the samples obtained from the cold room at Oniyanrin, Ibadan North LGA, 20 (33.3 %) were identified as *S. aureus*, 12 (20.0 %) as *L. monocytogenes*, 8 (13.3 %) as *Salmonella* spp. and 4 (6.7 %) as *E. coli*. (Table 1).

Overall, the prevalence of *L. monocytogenes* was significantly associated with sampling location ($P = 0.008$), with meat from Ojoo and Oniyanrin being about 9 (OR = 9.33, 95 % CI: 1.87—46.68, $P = 0.007$) and 3.5 (OR = 3.5, 95 % CI: 0.65—18.98, $P = 0.146$) times, respectively; more likely to be contaminated than those from Idi-Ose. Similarly, the prevalence of *S. aureus* was significantly associated with sampling location ($P = 0.000$), with meat from Ojoo and Oniyanrin being about 100 (OR = 0.01, 95 % CI: 0.002—0.065, $P = 0.000$) and 12.5 (OR = 0.08, 95 % CI: 0.021—0.282, $P = 0.000$) times, respectively less likely to be contaminated than that from Idi-Ose. However, sampling location was not significantly associated with the prevalence of *Salmonella* spp. ($P = 0.435$) and *E. coli* ($P = 0.117$).

Antibiotics susceptibility test

All of the isolates exhibited total resistance to seven of the 14 antibiotics used and these included ampicillin (10 µg), ceftazidime (30 µg), cefuroxime (30 µg), cotrimoxazole (25 µg), chloramphenicol (10 µg), cloxacillin (5 µg) and erythromycin (5 µg) (Table 2). In addition, 84.2 %, 100.0 %, 83.3 % and 50.0 % of all the *S. aureus*, *L. monocytogenes*, *Salmonella* spp. and *E. coli* isolated were respectively susceptible to ciprofloxacin (5 µg) while 84.2 %, 100.0 %, 77.8 % and 50.0 % were susceptible to ofloxacin in the same order (Table 2). Based on the proportion of susceptibility, *E. coli* exhibited the least susceptibility, being susceptible to only two antibiotics, followed by *Salmonella* spp. to three antibiotics, and then *L. monocytogenes* to four; the highest being *S. aureus* with total susceptibility to seven antibiotics (Table 2). Based on the locations of the different cold rooms from where the samples were collected, similar susceptibility and resistance patterns were obtained for the isolates (Table 3).

Table 1. Prevalence of foodborne pathogens from meat swabs samples obtained from cold rooms across Ibadan metropolis

Location	*S. aureus* Number (%)	*L. monocytogenes* Number (%)	*Salmonella* spp. Number (%)	*E. coli* Number (%)
Idi Ose	52 (86.7)	4 (6.7)	16 (26.7)	8 (13.3)
Ojoo	4 (6.7)	24 (40.0)	12 (20.0)	0 (0.0)
Oniyanrin	20 (33.3)	12 (20.0)	8 (13.3)	4 (6.7)
Total	**76 (42.2)**	**40 (22.2)**	**36 (20.0)**	**12 (6.7)**

Table 2. Antibiotic susceptibility profiles of foodborne pathogens isolated from stored meat from major cold rooms in Ibadan

	S. aureus [n = 76]			*L. monocytogenes* [n = 40]			*Salmonella* spp. [n = 36]			*E. coli* [n = 12]		
Susceptibility patterns* [%]	S	I	R	S	I	R	S	I	R	S	I	R
Ciprofloxacin [5 µg]	84.2	5.3	10.5	100.0	0.0	0.0	83.3	0.0	16.7	50.0	8.3	41.7
Ofloxacin [5 µg]	84.2	4.0	11.8	100.0	0.0	0.0	77.8	0.0	22.2	50.0	33.3	16.7
Augmentin [30 µg]	65.8	2.6	31.6	0.0	15.0	85.0	0.0	33.3	66.7	0.0	33.3	66.7
Nitrofurantoin [300 µg]	2.6	2.6	94.7	80.0	0.0	20.0	0.0	11.1	88.9	0.0	16.7	83.3
Ampicillin [10 µg]	0.0	0.0	100.0	0.0	5.0	95.0	0.0	25.0	75.0	0.0	41.7	58.3
Ceftazidime [30 µg]	0.0	0.0	100.0	0.0	12.5	87.5	0.0	16.7	83.3	0.0	0.0	100.0
Cefuroxime [30 µg]	0.0	0.0	100.0	0.0	2.5	97.5	0.0	5.6	94.4	0.0	8.3	91.7
Gentamicin [10 µg]	73.7	7.9	26.3	65.0	7.5	27.5	0.0	22.2	77.8	0.0	8.3	91.7
Cotrinazole [25 µg]	0.0	10.5	89.5	0.0	25.0	75.0	0.0	2.8	97.2	0.0	25.0	75.0
Chloramphenicol [10 µg]	0.0	7.9	92.1	0.0	20.0	80.0	0.0	0.0	100.0	0.0	50.0	50.0
Cloxacillin [5 µg]	0.0	19.7	80.3	0.0	27.5	72.5	0.0	13.9	86.1	0.0	16.7	83.3
Erythromycin [5 µg]	0.0	6.6	93.4	0.0	15.0	85.0	0.0	5.6	94.4	0.0	8.3	91.7
Streptomycin [10 µg]	13.2	3.9	82.9	0.0	22.5	77.5	22.2	2.8	75.0	0.0	33.3	66.7
Tetracycline [10 µg]	21.0	13.2	65.8	0.0	7.5	92.5	0.0	0.0	100.0	0.0	0.0	100.0

*—Susceptible; I—Intermediate; R—Resistant

Table 3. Percentage susceptibility of foodborne pathogens obtained from stored meat according to the different cold rooms in Ibadan

Cold room location	Idi-Ose				Ojoo			Oniyanrin			
Foodborne pathogens	***S. aureus*** [n=52]	***L. monocyto-genes*** [n=4]	***Salmonella*** [n=16]	***E. coli*** [n=8]	***S. aureus*** [n=4]	***L. monocyto-genes*** [n=24]	***Salmonella*** [n=12]	***S. aureus*** [n=20]	***L. monocyto-genes*** [n=12]	***Salmonella*** ([n=8]	***E. coli*** [n=4]
Antibiotic	%	%	%	%	%	%	%	%	%	%	%
Ciprofloxacin [5 µg]	80.8	100.0	87.5	75.0	100.0	100.0	66.7	90.0	100.0	100.0	0.0
Ofloxacin [5 µg]	92.3	100.0	87.5	75.0	75.0	100.0	50.0	65.0	100.0	100.0	0.0
Augmentin [30 µg]	73.1	0.0	0.0	0.0	50.0	0.0	0.0	50.0	0.0	0.0	0.0
Nitrofurantoin [300 µg]	0.0	100.0	0.0	0.0	50.0	75.0	0.0	0.0	83.3	0.0	0.0
Ampicillin [10 µg]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ceftazidime [30 µg]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cefuroxime [30 µg]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gentamicin [10 µg]	76.9	100.0	0.0	0.0	100.0	58.3	0.0	60.0	66.7	0.0	0.0
Cotrinazole [25 µg]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chloramphenicol [10 µg]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cloxacillin [5 µg]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Erythromycin [5 µg]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Streptomycin [10 µg]	19.2	0.0	37.5	0.0	0.0	0.0	16.7	0.0	0.0	0.0	0.0
Tetracycline [10 µg]	30.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

DISCUSSION

Foodborne diseases occur worldwide, including those acquired through the consumption of contaminated meat [3]. Microbial contaminated meat and meat products especially due to bacterial pathogens remain the most serious meat safety issue resulting in immediate consumer health problems and product recalls from the marketplace [26, 43]. The results of this study revealed heavy microbial contamination in meat from the cold rooms in Ibadan with an associated high level of resistance to major antibiotics. These findings indicate that raw meat remains a potential medium for the multiplication of foodborne pathogens, particularly bacteria and a likely source of antibiotic resistance development in human consumers. This observation is of serious public health concern since meat is a major source of animal proteins and most consumers do not translate their knowledge of food safety when handling meat into practice. Thus, they are exposed especially to the toxins produced by pathogens which are not readily inactivated by heat [1, 4]. Microbial contamination of meat and meat products renders the meat unwholesome and unfit for human consumption [20]. Meanwhile, most of such contaminations often occur at major critical points in the field, during harvesting, processing or distribution, or in retail markets, or foodservice facilities [19, 22, 35, 40].

The high prevalence of the foodborne pathogens isolated from stored meat in this study was unexpected considering the fact that the samples were taken from cold room facilities which were expected to be at the freezing point. A previous report shows that most food pathogens including *S. aureus, Salmonella, E. coli* do not survive sufficiently low temperatures [29], although *L. monocytogenes* may grow at temperatures as low as –2 °C. The observations in this study are therefore suggestive of poor operation and temperature holdings of cold rooms being used in preserving meat in the study area. The fact that the cold room owners were also meat traders themselves made them a sandwich between being informal meat traders and cold room owners. As a result, the meat preserved in the cold rooms were frequently exposed to thawing and freezing cycles when displayed for buyers who patronized the cold rooms. The resultant cycles of thawing and freezing could enhance microbial multiplication. These micro-organisms on the meat could proliferate as the temperature approaches the optimum range for their growth in the open air. This connotes a matter of serious public health concern to unsuspecting buyers since a visibly frozen meat in this case does not necessarily imply that it is safe and wholesome. Poor preservation of food including meat and meat products remains a challenge in Nigeria and it is responsible for untold post-harvest losses as well as increasing foodborne illnesses [9].

Notwithstanding the prevalence of 42.2 %, 22.2 %, 20.0 % and 6.7 %, respectively, observed for *S. aureus*, *L. monocytogenes, Salmonella* spp. and *E. coli* in this study, higher prevalence has been reported from informal meat markets. For instance, a report [38] indicated prevalence of 94.4 %, 88.8 %, 84.7 % and 69.4 % for *S. aureus, L. monocytogenes, Salmonella* spp. and *E. coli*, respectively, in both informal and formal markets in the same part of Ibadan metropolis where the present study was carried out. Adeyanju and Ishola [2] also obtained prevalence of 33.3 % and 43.4 % for *Salmonella* and *Escherichia coli*, respectively in poultry meat in Ibadan, while another study [15] revealed 96.3 % prevalence of *S. aureus* in raw beef in Lafia Metropolis, Nigeria. A higher prevalence of 95.8 % than what was observed in this study (22.2 %) was reported for *L. monocytogenes* in poultry meat by Ishola et al. [31] in Oyo State.

The varying prevalence notwithstanding, the high frequency of these pathogens observed in this study is indicative of unhygienic handling operations of meat during and post-processing. The high prevalence of *S. aureus* is particularly worrisome since the toxin it produces is heat-stable. This pathogen is resistant to high sodium chloride concentration and produces enterotoxins that can withstand high temperature, causing vomiting and diarrhoea when ingested [12]. As previously reported, a staphylococcal toxin dose of less than one microgram in contaminated food can produce symptoms of staphylococcal intoxication [23]. In the same vein, listeriosis has been associated with the consumption of undercooked raw foods contaminated with *L. monocytogenes* [13]. This disease often results in admission to intensive-care units, making *L. monocytogenes* the third most costly foodborne pathogen in the USA per case in 2010, after *Clostridium botulinum* and *Vibrio vulnificus* [41]. In 2012, an estimated annual cost of *L. monocytogenes* in the USA was put at US$ 2—6 billion [28]. The prevalence of *Salmonella* spp. in this study is also worthnoting, especially because of its implication in foodborne salmonellosis in humans, due to ingestion of *Salmonella*

cells. Previous findings have linked human salmonellosis outbreaks to consumption of contaminated meat [18, 36]. Also, *Escherichia coli* has been associated with traveller's diarrhoea and haemorrhagic colitis. Its presence in food is therefore considered a threat to human health, and an indication of gross contamination by human and/or animal faecal matter [7, 44].

Further, the level of resistance to antibiotics exhibited by most of the foodborne pathogens isolated in this study calls for serious attention. Antibiotics are commonly used around the world to cure diseases caused by bacteria, but as the World Health Organisation and other international bodies have pointed out; the global increase in antibiotics resistance is a rapidly worsening problem [14]. Since antibiotics are also an essential part of modern medicine as prophylactic treatment, the rising resistance of bacteria presents even more of a danger. As observed in this study, all the isolates exhibited total resistance to ampicillin (10 µg), ceftazidime (30 µg), cefuroxime (30 µg), cotrimoxazole (25 µg), chloramphenicol (10 µg), cloxacillin (5 µg) and erythromycin (5 µg). In a study on the emergence of a new antibiotic resistance mechanism in India, Pakistan and the UK [33], 36 isolates of *E. coli* obtained were highly resistant to all antibiotics except tetracycline and colistin. This is in line with the observation in this study as the *E. coli* isolates were resistant to all, but ciprofloxacin and ofloxacin among the antibiotics used. In a study on the characterization of antimicrobial resistance of foodborne *Listeria monocytogenes* [17], resistance to linezolid, ciprofloxacin, ampicillin and rifampicin, trimethoprim/sulphamethoxazole, vancomycin and tetracycline were observed among some of the isolates. G o m b a et al. [25] in their study also revealed that the *Salmonella* isolates assessed were resistant to most of the antibiotics.

On the other hand, the majority of the *S. aureus* strains in our study were susceptible to ciprofloxacin, ofloxacin and gentamicin. This was similar to the observation of B e r n a r d et al. [11] in their study to determine the antibiotic sensitivity of *S. aureus* strain responsible for community-acquired skin infections where only 0.5 % of the isolated strains showed resistance to gentamicin. This plausibly indicated that the resistance of *S. aureus* to gentamicin was still generally low.

The above findings notwithstanding, this study has some limitations. One, only three cold room facilities were used as using more facilities would have probably given more insights into the subject. Two, operations and activities of the cold room facilities studied were not investigated; this would have helped determine the factors responsible for the high level of microbial contamination observed.

CONCLUSIONS

Despite the above limitations, this study revealed that the meats preserved in commercial cold rooms in Ibadan metropolis were heavily contaminated with foodborne pathogens, including *S. aureus, L. monocytogenes, Salmonella* spp. and *E. coli* with serious public health implications to the consumers. The high level of antibiotic resistance exhibited by most of the foodborne pathogens isolated is a matter of grave concern to the health of animals as well as meat consumers. The high microbial contamination might be due to exposure to environmental pathogens from multiple handling and during frequent cycles of thawing and freezing of stored meat as well as possible inadequate freezing. Again, indiscriminate use of antibiotics in livestock animals in general by farmers might explain the observed high antibiotic resistance observed among the foodborne pathogens. Improved pre- and post-processing meat handling is advocated. Approaches to lower or control indiscriminate use of antibiotics in animals are also urgently required. There is a need to investigate structural facilities and the management of cold rooms in Nigeria and other developing countries towards ensuring wholesome and safe meat for ultimate human health. Again, there is a need for improved food preservation methods to mitigate the potential health hazards that are associated with the current practice in the study area.

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SEARCH FOR THE OCCURRENCE OF *CLOSTRIDIUM DIFFICILE* AND *CLOSTRIDIUM PERFRINGENS* IN PIGS WITHIN ZARIA AND ENVIRONS, IN KADUNA STATE, NIGERIA

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ABSTRACT

Animals have been known to be the main study subjects when investigating the epidemiology of zoonotic Gram-positive *Clostridium difficile* and *Clostridium perfringens*. This cross-sectional study was aimed at determining the occurrence of *C. difficile* and *C. perfringens* in pigs as well as the associated risk factors within Zaria and environs, in Kaduna State of Nigeria. A pre-sampling survey led to the selection of Shika, Samaru and Ungwan Mangu in the pig farming communities of Zaria and environs in Kaduna North as the study sites. Rectal swabs from 132 pigs were obtained and anaerobically cultured in fluid thioglycolate and further grown on reinforced clostridia agar. The colonies obtained were sub-cultured in *Clostridium difficile* moxalactam norfloxacin agar and reinforced clostridia agar containing egg yolk tellurite. *C. difficile* was not detected. However, *C. perfringens* was detected at a prevalence of 16.7 % (22/132). Isolates were tested for their susceptibility to 13 antimicrobials. Only 1 isolate (4.55 %) demonstrated susceptibility to vancomycin, gentamicin, chloramphenicol and erythromycin. Of the bivariate analyses of the risk factors studied, only the type of piggery and pig management were statistically significant ($P < 0.05$) for *C. perfringens*. Therefore, it should be recognized that there is a need for pig farmers to be enlightened about this pathogen and its prevention through good management practices and hygiene.

Key words: antibiotic susceptibility; *Clostridium difficile*; *Clostridium perfringens*; pig management

INTRODUCTION

Clostridium difficile (now reclassified as *Clostridioides difficile* [23, 32] and *Clostridium perfringens* are Gram positive, anaerobic spore-forming bacteria [4, 47]. They are among the most common causes of broad spectrum antibiotic associated diarrhoea (AAD) in humans and other animals [33, 39, 50]. The transmission of both *Clostridium* spp. among most animal species is mostly via faeco-oral and/or from the environment. The transmission is primarily due to their characteristics clostridia spores which

can overcome the unhospitable acidic condition of the gastrointestinal tract [29, 46].

After gaining passage to the large intestine, under proper conditions, clostridia organisms are able to germinate and colonize the large intestine. *C. difficile* produces large glucosylating exotoxins toxin A (TcdA) and the enterotoxin toxin B (TcdB), which results in the characteristic pathology of *C. difficile* infections. These toxins are considered the major virulent factors associated with *C. difficile* disease [9, 15, 26]. Some strains of *C. difficile* produce an adenosine diphosphate ribosylating binary toxin; however, the role of this toxin in the pathogenesis of the disease associated with *C. difficile* is yet to be elucidated [15, 35].

C. perfringens is classified into seven strains: A, B, C, D, E, F and G [22, 41]. It has been reported that the clinical signs of clostridial diarrhoea in pigs are similar to those of several other enteric diseases [38] caused by enterotoxigenic *E. coli* [7, 10], *Cryptosporidium* spp. [24], *Salmonella enterica* [18] and *Staphylococci* spp. [27] making it difficult to determine its true prevalence in pigs [11]. The absence of routine clinical diagnoses for clostridial spp. diarrheal cases results in little attention being given to *Clostridium difficile* and *Clostridium perfringens* infections in pigs within Zaria and environs, in Kaduna State of Nigeria. This research was stimulated in order to provide a baseline data on the occurrence, antimicrobial susceptibility of *C. difficile* and *C. perfringens* in pigs and their associated risk factors within Zaria and environs, of Kaduna State, Nigeria.

MATERIALS AND METHODS

Study design

A cross sectional study design was used in this research from April—August, 2018. The snowball sampling technique (majority of residents are of Islamic faith) was used to identify owners with pigs in the settlements of Shika, Samaru and Ungwan Mangu. These three settlements were selected based on the presence and availability of pigs where faecal samples could be collected from pigs and questionnaires administered to the farm's owners or attendants.

This study was carried out in Zaria, in Kaduna state of Nigeria, which is positioned between latitude 11° 07"N and 11°12' N. and longitude 07°41'E. Zaria is in the centre of northern Nigeria and located on a plateau 2200 feet above sea level.

Zaria is characterized by a tropical climate, a monthly mean temperature ranging from 13.8—36.70 °C and an annual rainfall of 1092.8 mm. Dry season farming is the second most prevalent agricultural activity in Zaria with vegetables being the common produce, but in some cases fruits are sandwiched among the cereal crops [1]. It has an estimated population of 408,198 people (NPC, 2006). Animals being raised are mostly ruminants [3], although the pig population in Kaduna State has been estimated to be 249,651 [36] even though no recent census has been conducted.

Sample size determination

The sample size was determined using the formula by Thrushfield [44] at 95% confidence interval. Subsequently, a prevalence of 8.6% by Norman et al. [31] and a sample size of 121 was obtained. Later, 9.09% of 121 was added to reduce the sampling error and accommodate additional samples, thereby increasing the sample size to 132.

Isolation and culturing

Rectal swabs (n = 132) were cultured in fluid thioglycollate (Merck, KGaA, Germany) which were incubated aerobically at 37 °C for 24 hrs. The cultures were streaked onto reinforced clostridia agar and incubated anaerobically. The suspected colonies were then sub-cultured onto two media, first *Clostridium difficile* Agar Base (Oxoid, CM0601; Oxoid Ltd., Hampshire, United Kingdom) containing Moxalactam, Norfloxacin and Cysteine hydrochloride as selective supplements (CDMN, SR0173; Oxoid Ltd., Hampshire, United Kingdom) with 7% v/v horse blood and reinforced clostridial agar medium containing egg yolk tellurite. The streaked plates were incubated anaerobically using an anaerobic jar incorporated with a gas generating kit (Thermoscientific, AnaeroGenTM 3.5L, AN0035A, Oxoid Ltd, Wade Road, Hants, UK) at 37 °C for 48—72 hrs according to a modified method of Tizhe et al [45]. The suspected isolates of *C. difficile* were viewed for grey white colonies on CDMN agar while those grown on blood agar were viewed under ultraviolet light for the characteristic morphology of *C. difficile* with yellow green fluorescence while suspected isolates of *C. perfringens* were viewed for black colonies on reinforced clostridial agar while those grown on blood agar were viewed for yellow grey colonies and a double zone of haemolysis on blood agar.

Biochemical typing of *Clostridium* isolates

Suspected isolates were subjected to the following conventional array of biochemicals: the nitrate reduction test, lecithinase test, sugars (glucose, lactose, mannitol, inositol, dulcitol, arabinose, sucrose and rhamnose), L-arginine test, catalase test, triple sugar iron test, urease test, indole test and methyl red Voges Proskaeuer test [5, 17, 20].

Antibiotic sensitivity testing

The susceptibility testing was performed using the Kirby-Bauer disk diffusion method [6]. The antibiotics used were those commonly used by farmers: vancomycin (30 µg), gentamicin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), ciprofloxacin (5 µg), streptomycin (10 µg), oxacillin (1 µg), clindamycin (2 µg), cefoxitin (30 µg), penicillin (10 µg), sulphamethazole-trimethoprim (25 µg), amoxycillin-clavulanic acid (30 µg) and imipenem (10 µg). The zones of inhibition were measured and compared with the Clinical and Laboratory Standards Institute [13] and the European Committee on Antibiotic Susceptibility Testing [16] guidelines.

Questionnaire administration

A structured questionnaire (73) was administered to the owners of the pig farms that were sampled to obtain information on pig management, attitude and practices as it related to the risk of possible acquisition of *C. difficile* and *C. perfringens* infections in pigs.

Data analyses

The statistical Package for Social Sciences (SPSS VERSION 21.0) was used to analyse the data obtained. The Fisher's exact test was used to show associations between *C. perfringens* and risk factors. The risk factors were assessed using regression and odd ratio at 95 % confidence interval. The absence of a risk factor was considered as a reference category for odds ratio. The values of $P < 0.05$ were considered significant. The data obtained were presented in the subsequent tables.

RESULTS

There was no *C. difficile* isolated from pigs but instead, only *C. perfringens* were isolated. Table 1 shows the number of animals that were positive for *C. perfringens*, from Shikah and Samaru. None were positive from Ungwan Mangu, while out of all of the pigs sampled (132), only 22 (16.7 %) pigs were positive for *C. perfringens*.

As to the antibiotic susceptibility test carried out for the various isolates, only 1 isolate (4.6 %) was susceptible. All of the other isolates were resistant to: vancomycin, gentamicin, chloramphenicol and erythromycin while there was 100 % resistance to ciprofloxacin, streptomycin, oxacillin, clindamycin, cefoxitin, penicillin, imipenem, sulphamethazole-trimethoprim and amoxycillin-clavulanic acid (Table 2).

Possible environmental risk factors associated with the presence of *C. perfringens* infections

Based on the analyses of the responses to the questions on the type of piggery in relation to the presence of *C. perfringens*, 3 (4.2 %) of the farmers having the backyard type of piggery had the *C. perfringens* in the faeces of their pigs, while 68 (95.2 %) had an absence of *C. perfringens* in the faeces of their pigs, and 2 (100 %) of those with established farms had an absence of *C. perfringens* from the rectal swabs collected from their pigs. Also, the response to questions on pig management in relation to *C. perfringens* showed that the pigs which were reared under intensive, semi-intensive and extensive to be 14.3 %, 1.6 % and 100 % had the presence of *C. perfringens* while there was an absence of *C. perfringens* in pigs of those who were raised under intensive 6 (85.7 %), semi-intensive 63 (98.4 %) methods (Table 3).

Table 1. Distribution of *C. perfringens* isolates based on the location and gender in Zaria and environs

Sampled area	Sex	No. of pigs sampled	No. of pigs positive (%)
Shikah	Male	30	13 (43.3)
	Female	20	6 (30.0)
	Total	50	19 (38.0)
Samaru	Male	42	2 (4.7)
	Female	20	1 (5.0)
	Total	62	3 (4.8)
U/Mangu	Male	16	0.0
	Female	4	0.0
	Total	20	0.0
Total		**132**	**22 (16.7)**

Table 2. Susceptibility of *C. perfringens* isolates from pigs to routinely used antimicrobials in Zaria and environs

Antimicrobial agent (concentration)	Susceptible (%)	Intermediate (%)	Resistant (%)
Vancomycin (30 µg)	1 (4.5)	0 (0.0)	21 (95.45)
Gentamicin (30 µg)	1 (4.55)	0 (0.0)	21 (95.45)
Chloramphenicol (30 µg)	1 (4.55)	0 (0.0)	21 (95.45)
Erythromycin (15 µg)	1 (4.55)	0 (0.0)	21 (95.45)
Ciprofloxacin (5 µg)	0 (0.0)	0 (0.0)	22 (100)
Streptomycin (10 µg)	0 (0.0)	0 (0.0)	22 (100)
Oxacillin (1 µg)	0 (0.0)	0 (0.0)	22 (100)
Clindamycin (2 µg)	0 (0.0)	0 (0.0)	22 (100)
Cefoxitin (30 µg)	0 (0.0)	0 (0.0)	22 (100)
Penicillin (10 µg)	0 (0.0)	0 (0.0)	22 (100)
Imipenem (10 µg)	0 (0.0)	0 (0.0)	22 (100)
Sulphamethazole-trimethoprim (25 µg)	0 (0.0)	0 (0.0)	22 (100)
Amoxycillin-clavulanic acid (30 µg)	0 (0.0)	0 (0.0)	22 (100)

Table 3. Response of pig owners to questions on the type of piggery and pig management in relation to presence/absence of *C. perfringens* in Zaria and environs

Variable	Present (%)	Absent (%)	Odd ratio	C. I value	P value
Type of piggery backyard	3 (4.2)	68 (95.2)	0.44	0.20—0.95	0.001
Established pig management system	0 (0.0)	2 (100)	REF		
Intensive	1 (14.3)	6 (85.7)	0.47	0.08—1.44	0.001
Semi-intensive	1 (1.6)	63 (98.4)	1	0.14—13.38	
Extensive	1 (100)	0 (0.0)	REF		

C.I—95 % confidence interval. Fisher's exact test; REF—Reference category

Table 4. Response of pig owners to questions on the keeping of other animals and allowing other boars to service their sows in relation to presence/absence of *C. perfringens* in Zaria and environs

Variable	Present (%)	Absent (%)	Odd ratio	C. I value	P value
Those who keep animals other than pigs					
Yes	2 (4.4)	43 (95.6)	1.2	0.10—14.0	1
No	1 (3.7)	26 (96.3)	REF		
Those who carry their pigs to be serviced by other boars outside the farm					
Yes	3 (4.8)	59 (95.2)	0.95	0.90—1.0	0.68
No	0 (0.0)	11 (100)	REF		

CI—95 % confidence interval (Fishers exact test); REF—Reference category

Table 5. Type of piggery and management systems of pigs within Zaria and environs

Variables	Frequency	Percentage (%)
Type of piggery		
Backyard	71	97.3
Established	2	2.7
Pig management		
Intensive	7	9.6
Semi-intensive	65	89
Extensive	1	1.4

Respondents who noted yes (4.4 %) and no (3.7 %) to questions on keeping animals other than pigs had *C. perfringens* from the rectal swabs collected from the pigs even though there was no statistical significance (OR-1.2 p value 1.0), while respondents who said yes (4.8 %) to questions on carrying their pigs to be service by other pigs outside the piggery had *C. perfringens*, while 95.2 % had an absence of *C. perfringens* even though no statistical significant association was evident (Table 4).

Table 5 shows the frequency of pig holders within Zaria who had the backyard (97.3 %) and established (2.7 %) type of piggery, as to pig management; 9.6 % practiced the intensive pig management, while 89 % practiced the semi-intensive management and 1.4 % practiced the extensive management.

DISCUSSION

In this study, the isolation of *C. difficile* was attempted, but none was recovered. It has been shown that the prevalence of *C. difficile* carriage in swine drops dramatically with age [28, 49] and perhaps that may have been the reason for the absence of *C. difficile* in this study, as most of the pigs sampled were adults of reproductive age. However, *C. perfringens* was isolated successfully. The shedding of these two organisms in faeces constitutes a risk factor to piglets. Ferreira et al. [19] established a high prevalence (58.8 %) of *C. perfringens* from the 90 pigs sampled. This difference in the prevalence between *C. difficile* and *C. perfringens* may have been due to different practices employed by the pig owners or genetic factors of the particular breed of pigs. *Clostridia* spp. are usually the first colonizers of the piglet gastrointestinal tract; thereby predisposing the piglets to infection which might later lead to diarrhoea [42].

Antibiotics are used as therapeutics, growth promoter and as a prophylaxis [14]. The 22 isolates in this study were subjected to 13 antibiotics, of which only one isolate was susceptible to gentamicin, chloramphenicol and erythromycin. Perhaps a different strain of *C. perfringens* was present among the isolates. Antimicrobial resistance among anaerobes has consistently been on the increase in the past 3 decades and the susceptibility of anaerobic bacteria to antimicrobial agents has become less predictable especially among anaerobes, such as *Clostridium* spp., that were previously very susceptible [8]. This increase resistance makes

the choice of appropriate empirical therapy even more difficult in a developing country like Nigeria since a lot of farmers have no knowledge of *C. perfringens*, but tend to attribute diarrhoea caused by this bacterium to other bacteria and parasites.

Our work is at variance with the reports of van den Bogaard et al. [48] and Ngamwongsatit et al. [30] who reported the resistance of *C. perfringens* isolates from pigs to chloramphenicol and erythromycin. The reason for the susceptibility seen in our study might be due to the fact that the resistance had not yet developed in this isolate. Also, in a recent study in Lagos, Nigeria, *C. perfringens* isolated from food and human faecal specimens were resistant to erythromycin, in which the authors reported that some of the isolates might have originated and transmitted through faecal contamination of food [12].

As to the penicillin and clindamycin resistance, it has been reported by the CLSI [13] that some isolates of *C. perfringens* are resistant to these two antibiotics which was observed in our study, even though pig farmers use most of the antibiotics for empirical therapy against other known pathogens that may mask or confound the clinical signs of *C. perfringens* with rare visits to the veterinarian for confirmation of the causal organism (based on personal communication with most of the farmers). Antimicrobial resistance reported from livestock are mostly of anthropogenic origin [2]. A lot of resistance studies to a wide range of isolates tested against antimicrobials belonging to different classes of antibiotics in Nigeria which shows that almost in all instances, resistant pathogens were recovered from livestock (pigs, goats, cattle, sheep, chickens and camels). Also, the most available information on resistance in Nigeria are products of "individuals" effort studies [2], which perhaps is the reason why most researchers cannot go further to find out the sources of resistance. Reports of resistance to amoxycillin-clavulanic acid, vancomycin, imipenem, penicillin, chloramphenicol and ceftriazone (same group with cefoxitin) antibiotics have also been reported in isolates of *C. perfringens* in pigs from Thailand, Canada and Brazil [37, 40, 43].

Food producing animals are linked to humans through the food chain and shared environment. The possibility exist that antimicrobials residues will always be present in meats [21, 25, 34] of these pigs at the time of sale and consumption, and that is why Chukwu et al. [12] said that "there is need for periodic antimicrobial susceptibility testing and surveillance to detect geographic and temporal changes in the resistance trends of *C. perfringens*".

From our study, there were more backyard piggeries than established farms, and this might have been due to the fact that pig farmers need to keep a close watch over their pigs. Also, most of the pig owners might not be rich enough to own landed properties and the funds to run an establish farm. Most of these piggeries were located close to toilets (pit toilets) and with the bathroom drainages passing close to the piggeries partially serving as water for the pigs. This may serve as reason why pigs were being infected with *C. perfringens*. While sampling, it was observed that some farmers threw vegetables along these drainages and the pigs consumed these vegetables like corchorus stocks (ayoyo) and cabbage leaves. Perhaps this might be the reason for the strong association between the type of piggery and *C. perfringens*. Chukwu et al. [12] reported the presence of *C. perfringens* in cabbage in Lagos state of Nigeria and also, *C. perfringens* has been reported in water [42]. During the course of sampling it was discovered that most of the piggeries were not cemented; perhaps, another reason for the presence of *C. perfringens*, since one of their principal habitat is the soil [25].

Pig management has always been an issue in areas where pigs are raised because of the damage they cause to crops in the rainy season. One of the sampled areas that had the highest prevalence of *C. perfringens* did keep their pigs under strict intensive management. However, during the course of sampling it was observe that the association between the pigs and other animals in the environment was not different from other pigs that were being manage under the semi-intensive and extensive system. As a result, this could have contributed to the prevalence of *C. perfringens* through the faeces of the other animals that associate with the pigs. A lot of pig farmers practiced the semi-intensive management, in which the pigs were allowed to scavenge for food. These pigs returned in the evening and were given watery cereal bran. Prior knowledge of where the animals had been was unknown to the owners. This act of scavenging and consumption of contaminated feed and faeces may result in *C. perfringens* infections observed in this study, even though $P<160.05$, but the strength of the association between pig management and *C. perfringens* was weak.

CONCLUSIONS

C. difficile was not isolated but *C. perfringens* was found with a prevalence of 16.7 %. Most of these isolates were resistant to the antibiotics tested. Only 1 isolate (4.55 %) was susceptible to vancomycin, erythromycin, gentamycin and chloramphenicol. The environmental risk factors associated with *C. perfringens* included the type of piggery and pig management. The pre-dominance of smallholder/family pig farming has made it difficult to focus attention on the epidemiology of clostridial diseases in the pig industry here in Zaria and environs, Kaduna State, Nigeria.

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MICRONUCLEUS ASSAY IN ENVIRONMENTAL BIOMONITORING

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ABSTRACT

Nowadays many chemicals are widely used in agriculture to ensure high crop yields or in veterinary/human medicine to cure diseases. After their improper usage they may contaminate the environment, persist in it and adversely affect both the target and/or the non-target organisms. One of the ways to detect the occurrence of chemicals in the environment is to assess their impact on aquatic and farm animals; both are directly or indirectly exposed via their feed and water. The micronucleus assay is a standardly used cytogenetic test for the simultaneous detection of clastogenic and aneugenic agents. Additionally, cytotoxic effects are also assessed by analysing the proliferation changes using the cytokinesis-blocked proliferation index. The occurrence of micronuclei is analysed in many types of cells like the peripheral blood cells, bone marrow or cell lines according to standards for micronuclei detection. The analysis of published results has shown that the micronucleus assay is, together with the chromosomal aberration test, one of the most often used test in genotoxicity assessment. Its results have contributed to reassessing the use of multiple chemicals available on the market. Moreover, it is a compulsory test before approving the chemical/pesticide for the market.

Key words: biomarker; cytotoxicity; genotoxicity; micronucleus; pesticide

INTRODUCTION

Micronuclei (MNs) are small, extranuclear chromatin corpuscles surrounded by a nuclear coat. They originate from acentric chromosomes or their fragments, that are lagging behind karyokinesis in anaphase and they are not part of the daughter nuclei at telophase [14, 23]. In 1959, for the first time, E v a n s et al. [12] described the use of MNs as biomarkers of DNA damage of the root tips of *Vicia faba* when the cytogenetic effects of neutrons (cobalt-60 gama-radiation and by fast neutrons) were tested.

In general, the presence of MN seems to serve as a signal for elimination. There are four possible ways of mi-

cronuclei elimination: degradation, reincorporation into the main nucleus, extrusion from the cell, and persistence in the cytoplasm; but there are two additional fates: the premature chromosome condensation/chromothripsis and the elimination of MN cells by apoptosis [23]. The degradation of MN was observed under specific situations in fixed primary and immortalized cells from humans and other animal species. Those MNs were induced by clastogenic/aneugenic agents and the type of degradation was variable (apoptic, autophagic, lysosomal). Autophagy represents one way of micronuclei degradation. It is a catabolic process when degradation of cellular components is caused by lysosomes [11]. Another possible fate of micronuclei degradation has been described by Rello-Varona et al. [37]. The authors found that MNs were removed by macroautophagy. A small percentage of MNs was localized inside autophagic vesicles and the collapse of the MN envelope observed could have been associated with DNA damage caused by those lysosomes. Then, the extrusion of the MNs has only been shown under very specific experimental circumstances; the MN can be extruded during a high concentrated cytoB-induced anucleation observed in mammalian cells [30] and/or micronuclei containing whole chromosomes can be extruded [39]. Then, chromotripsis (premature chromosome condensation) can occur. It causes massive genomic rearrangements resulting in the presence of highly mutated chromosomes within one cell cycle. A higher percentage of reincorporation was observed in MNs induced by aneugenic agents, but data from studies are not consistent, so general conclusions cannot be made. On the other hand, a very high percentage of MNs persists until the next mitosis without alteration [23].

The cytokinesis-block micronucleus assay (CBMN) is preferred using both *in vivo* and *in vitro* methods of MNs detection in various cell lines [14]. The author described the CBMN cytome assay as the comprehensive system for measuring DNA damage, cytostatic and cytotoxicity, where specifically once divided binucleated (BN) cells were scored. The occurrence of the MN (biomarker of chromosomal breakage and/or loss of whole chromosome), nucleoplasmatic bridges (NPB, biomarker of faulty DNA repair and/or telomere and fusion), nuclear buds (NB, biomarker of elimination of amplified DNA and/or complex DNA repair mechanism) are analysed. Moreover, the cytostatic effects are scored using the cytokinesis-blocked proliferation index (CBPI) which is the ratio between mono-, bi- and multinucleated cells and cytotoxic effects through the ratio of the necrotic and/or apoptotic cells.

The CBMN is used for monitoring of clastogenic (mainly induction of chromosomal fragments) and aneugenic (disruption of mitotic apparatus leading to mis-segregation either chromatids or whole chromosomes during mitosis) effects of the chemical agents as genotoxic damage. The MNs are also widely used as indicators of genomic instability and *in vivo* exposures by genotoxins in biomonitoring studies [23].

In this review we will summarize information and make conclusions whether the CBMN test is a suitable method for *in vivo* or *in vitro* genotoxicity testing in aquatic species and farm animals.

MATERIALS AND METHODS

The data were obtained by the analysis of previously published research involving methodology and analysis of the micronucleus assay and its application to genotoxicology assessment in water environments and farm animals. The authors "experiences" in the application of MN assay on bovine peripheral lymphocytes were also described and analysed in the article.

COMPARISON OF *IN VITRO* AND *IN VIVO* MICRONUCLEUS TEST

According to the OECD guidelines, the following two main types of MN assays are approved for chemical testing: the *in vivo* mammalian erythrocyte MN assay (OECD No. 474) [31] and the *in vitro* mammalian micronucleus test (OECD No. 487) [32]. Their summary and comparison can be found in Table 1.

SLIDE SCORING AND ANALYSIS

The criteria for scoring cells has been described in detail in the HUMN project [15] and CBMN cytome assay [14], and are used as standards for scoring the cells.

The microscopic analysis of each slide should be performed by two different scorers using identical microscopes and examining at 1000× magnification. The sam-

Table 1. Comparison of the *in vivo* mammalian erythrocyte MN test [31] and the *in vitro* mammalian cell MN test [32]

	***In vivo* mammalian erythrocyte MN test**	***In vitro* mammalian cell MN test**
Experimental models	mice, rats, other mammalian species	human, rodents
Samples	bone marrow, peripheral blood	peripheral blood cells-lymphocytes, cell lines
Solvents	water, physiological saline, methylcellulose solution, olive oil, corn oil	water, physiological saline, dimethyl sulfoxide (dmso), ethanol, acetone
Positive controls	ethyl methanesulphonate, methyl methanesulphonate, ethyl nitrosourea, mitomycin c, cyclophosphamide, triethylenemalamine, colchicines, viriblastine	methyl methanesulphonate, mitomycin c, 4-nitroquinoline-n-oxide, cytosine arabinoside, benzo(a)pyrene, cyclophosphamide, colchicines, viriblastine
Preparation of samples	bone marrow cells-humane euthanasia (femurs, tibias), peripheral blood – from tail vein or other blood vessel, cardiac puncture or large vessel	cell lines – harvested in culture medium, lymphocytes need mitogen stimulation (e.g. phytohaemagglutinin), with or without cytochalasin-b
Preparation for staining	stained for microscope (giemsa), fixed for flow cytometry (acridine orange/ hoechst 33258 + pyronin-y)	giemsa, acridine orange, hoechst 33258 + pyronin-y
Analysis/scoring cells	randomised manual evaluation (by microscope), automated systems (flow cytometrs, image analysis platforms, laser scanning cytometers)	by microscope or automated scoring systems (flow cytometry, laser scanning cytometry, image analysis)

ples should be ideally obtained and assessed from duplicate cultures. At first, the frequency of mono-, bi- and multinucleated viable cells, as well as apoptotic and necrotic in a minimum are analysed for each slide. Then the occurrences of micronuclei, nuclear bridges and buds are detected. The following data gives us: information about the nuclear division index/cytokinesis-block proliferation index, the number of apoptotic cells per 500 cells, the number of necrotic cells per 500 cells, and the frequency of BN cells containing MN, NPB, NB in at least 1000 BN cells [14, 15].

Nuclear division index (NDI) measures the proliferative status of the viable cell fraction,

$$NDI= (1 \times M_1 + 2 \times M_2 + 3 \times M_3 + 4 \times M_4)/N$$

where

M1—M4 represent the number of cells with 1—4 nuclei and N is the total number of viable cells scored (excluding necrotic and apoptotic cells). The NDI is a useful parameter for comparing the mitotic division of lymphocytes and cytostatic effects of agents examined in the assay.

Cytokinesis-block proliferation index (CBPI) is calculated according to S u r r a l l é s et al. [45] for the cell cycle delay and/or cytotoxicity induced by chemical agents,

$$CBPI = [1 \times M_1 + 2 \times M_2 + 3 \times (M_3 + M_4)]/n$$

where

M1—M4 represent the number of cells with 1—4 nuclei and n is the total number of scored cells.

Both NDI and CBPI reflect the dose dependency of cytotoxicity more accurately than only the percentage of BN cells do. The CBPI is similar to the NDI, expect that the NDI estimates the average number of nuclei per cell. The CBPI indicates the average number of cell cycles undergone by a given cell, which may be more relevant in the case of cytokinesis/blocked cells. The CBPI classifies both trinucleated and tetranucleated cells into the same category and equally considered being in their third cell cycle [45].

The anticipated results depend on several factors like: the culture conditions, level of exposure to genotoxic or cytotoxic agents and their potency, the composition of the medium, the genetic background, and the age and gender of the donor of the cells. For normal peripheral human blood lymphocytes, F e n e c h [14] assumed the following range of values: 30—60 % of BN cells; NDI: 1.3—2.2, 0—9 % of necrotic cells; 0—7 % of apoptotic cells; and 0—30 of MNi, 0—10 NPBs and 0—5 NBUDs per 1,000 BN cells, respectively.

According to the OECD protocol no. 474 for *in vivo* eryMN (erythrocyte micronucleus) test, all slides and samples for analysis should be scored according to its standards. The ratio among immature and total eryth-

rocytes (ery) should be determined for each animal by counting at least 500 erythrocytes from bone marrow and 2000 erythrocytes from the peripheral blood. Then, the incidence of the micronucleated immature erythrocytes should be scored in at least 4000 immature erythrocytes per animal [31].

For the *in vitro* cell MN test, it is recommended to score the cytokinesis-block proliferation index (CBPI) and/or Replication Index (RI) from at least 500 cells per culture to determine the cell proliferation and the cytotoxic and/or cytostatic activity using cytochalasin-B. Besides those, we can estimate other indicators of cytotoxicity, such as cell integrity, apoptosis, necrosis, metaphase counting, and the cell cycle. In test studies without cytochalasin-B, it is recommended to measure the Relative Population Doubling (RPD) and/or Relative Increase in Cell Count (RICC) to estimate the cytotoxic and cytostatic activity of the treatment. Moreover, other markers can be scored: cell integrity, apoptosis, necrosis, metaphase counting, proliferation index (PI), cell cycle, NPB, and NB.

The other possibility for the microscopic assessment of MN induction is the flow cytometry. The reliability of the flow cytometry is enhanced by two fluorescent staining methods of MNs. Both dyes are fluorescent specific. Ethidium monoazide bromide stains the chromatin of necrotic and mid/late stage apoptotic cells. After washing of cells and stripping of cytoplasmic membranes, they are incubated with SYTOX Green (RNase plus a pan-nucleic acid dye) which stain differentially the suspension of free nuclei and MNs [4].

At the same time, the usefulness of the *in vivo* MN assay scored by flow cytometry was tested. An immunochemical reagent, anti-CD71-FITC conjugate, together with propidium iodide were used to detect MNs in young polychromatic and normochromatic erythrocytes. In conclusion, it was found that flow cytometry can be used for the assessment of MN induction after acute and chronic exposures of rats to the aneugenic/clastogenic agents [5]. The flow cytometry method performed with Hoechst 33258 and propidium iodide is admissible for automated scoring in *in vivo* MN assay using rat bone marrow and peripheral blood [22]. Nowadays, automation of the MN assays with the use of imaging flow cytometry indeed represents „twenty-first century approach" and is able to identify and automatically score binucleated cells, micronucleated binucleated cells, mono-, multinucleated cells, NPB, NB, apoptotic and necrotic cells [38].

APPLICATION OF CBMN ASSAY IN AQUATIC ANIMALS

Cytogenetic assays on aquatic organisms are essential, as humans are exposed throughout their lifetime to many xenobiotics present in both the water and aquatic food. The MN test used for genotoxic biomonitoring of chemical compounds, pesticides and environmental pollutants has proven to be the most practical tool in genotoxicological assays; various aquatic species were used [8, 20, 51]. *In vivo* MN tests performed on fish and other aquatic species erythrocytes were used as useful tools in determining the potential genotoxicity of pesticides. Increased levels of micronuclei were found in rainbow trout *Oncorhynchus mykiss* after amoxicillin exposure at the highest concentration tested (320 $mg.kg^{-1}$) [2]. Another study evaluated the mutagenic potential of rotenone in the same fish and the results showed a significantly increased MN frequency in the four lowest concentrations tested: i. e. 0.06; 0.125; 0.187 and 0.25 $mg.l^{-1}$ [28]. Then, the potential genotoxic effect of glyphosate formulation Roundup® [8], atrazine-based formulation Gesaprim® [7], trifluralin-based formulation Treflan® [24], chlorpyrifos [1], malathion [25] and imidacloprid [54] were assessed in fish erythrocytes. In all, significantly elevated levels of MNs were detected.

On the other hand, atrazine as a single compound was found to be nongenotoxic, whereas atrazine-based formulation Gesaprim® showed genotoxic effects [7]. Also, the risk assessment of MN frequencies in flounder Platichthys flesus, herring Clupea harengus and eelpout Zoarces viviparus were investigated by Baršienė et al. [3]. They obtained data from different regions of the Baltic Sea in the years 2001 to 2011. Their MN analysis in erythrocytes showed that there were extremely high genotoxicity risk zones in many tested regions; the highest level of MN was found in flounder.

Not only fish were used as experimental models to assess the impact of environmental pollutants on freshwater ecosystems. The genotoxic effects of many pesticides accompanied with increased MN frequencies were detected in lizards (*Podarcis sicula*) [6], tadpoles of Chinese toad *Bufo gargarizans* [56], tadpoles of Indian frog *Euflictis cyanophlyctis* [55] and Montevideo tree frog *Hypsiboas pulchellus* [35]. Lajmanovich et al. [26] carried out erythrocyte MN tests on tadpoles of the common toad *Rhinella arenarum*. They exposed them to glufosinate-ammonium

and its commercial formulation Liberty® for 48 and 96 h at concentrations of 3.75; 7.5 and 15 mg.l^{-1}. A statistically significant increase of MN frequencies were detected only after 48 h treatment with Liberty® at the concentrations of 7.5 and 15 mg.l^{-1}. In another study, an increased frequency of micronuclei was observed in the tadpoles of *Hypsiboas pulchellus* [34]. They were exposed to imidacloprid-based formulation Glacoxan Imida® (12.5—37.5 mg.l^{-1}) for 48 and 96 h, but the genotoxic effects were seen only at the concentration of 25 mg.l^{-1} after the 96 h treatment.

The ambiguous results of flurochloridone-based formulations Twin Pack Gold® and Rainbow® genotoxicity were observed on tadpoles of toad *Rhinella arenarum* [29]. After exposure to Twin Pack Gold®, the MN frequency did not differ from the negative control, regardless of the tested concentrations and both exposure time, whereas Rainbow® caused an increase in the MN frequency only after 48 h treatment with the lowest concentration (0.71 mg.l^{-1}) tested. And also for the first time, a turtle *Trachemys callirostris* was used in the MN induction assessment [57]. This endemic species from northern Colombia was used to characterize DNA damage caused by contaminated environments.

Our water environments are dangerously polluted and the first contact with the pollutants are mainly fish, other aquatic and amphibian species. These results from the above mentioned studies indicated potential genotoxic effects of contaminants from the released and/or leaked chemical pollutants and used pesticides in agriculture near water sources. On the other hand, some studies showed inconclusive and ambiguous results, but there is still a need for further investigation using MN assays as a tool for genotoxicity monitoring.

APPLICATION OF CBMN ASSAY IN FARM ANIMALS

Pesticides are chemicals which help us in agriculture to control the pests, weeds or plant diseases, but on the other hand their extensive use leads in their accumulation in the environment and the possibility of potential hazards to animals´ health. A broad range of *in vivo* and *in vitro* cytogenetic assays have been used for genotoxicity assessment of pesticides. The predominantly used cytogenetic assays are the MN assay and the chromosomal aberration test performed on different experimental models and cell lines. Cattle and other farm animals like rabbits and chickens are mostly exposed to environmental pollutants via contaminated feed and water, which may result in the accumulation of some chemicals in the milk [13], meat and fat [27] used for primary consumption and food processing. Finally, negative health effects can be seen in animal offspring and humans after their consumption [33]. The methodology of MN assay performed on bovine peripheral lymphocytes has been described by G a l d í k o v á et al. [17]. Briefly, the peripheral blood from 2 healthy bulls were cultivated for 72 h at 37 °C in cultivation medium. Cytochalasin B was added 44 h after the initialization of the cultivation. The following time periods were used for pesticide treatments: 4, 24 and 48 h. Then, 1000 BNMN cells were scored for the MN induction and 500 cells for the CBPI analysis.

Neonicotinoid insecticides have been widely applied and acted as selective inhibitors of nicotinic acetylcholine receptors in the central nervous system of insects leading to accumulation of acetylcholine and subsequently to death [21]. The potential genotoxic effects of the insecticide thiacloprid and thiacloprid-based formulation Calypso® was evaluated after 24 and 48 h exposures. The results showed no significant increase in MN frequencies after any concentration tested (30, 60, 120, 240 and 480 μg.ml^{-1}) [17, 18]. On the other hand, both pesticides reduced the CBPI in a dose dependent manner after 24 h and a prolonged time of exposure as well. To elucidate the mechanism of action, the experiments with short-time treatment were also performed [41]. Unlike the previously published results, significant induction of micronuclei were observed accompanied with the CBPI reduction. The authors assumed that micronuclei induction was a temporary process in 4 h treatment compared to prolonged exposures due to micronuclei elimination during the cultivation process. Moreover, higher levels of apoptotic cells, cell cycle stop in G0/G1, recruitment of p53, and DNA double strand breaks were detected. Other authors evaluated the effects of the insecticides chlorpyrifos, cypermethrin and their mixture on bovine lymphocytes *in vitro* [16]. They found a higher incidence of MN and nuclear buds with a simultaneous decrease in CBPI after treatment with cypermethrin and the mixture. It is known they induce, oxidative stress, disturb synthesis of hormones and have a negative impact on reproduction.

The other group of tested pesticides represented triazole based fungicides. Their mode of action is based on ergos-

terol synthesis inhibition via lanosterol 14-α-demethylase inhibition [9]. Finally, fungal cell permeability is disturbed and slower cell growth occurs. An epoxiconazole-fenpropimorph-based fungicide formulation Tango® Super was tested in bovine lymphocytes using concentrations of 0.5, 1.5, 3, 6 and 15 μg.ml^{-1} for 4, 24 and 48 h exposures. The fungicide did not induce micronuclei production, but the cell proliferation was significantly decreased [10, 42]. Moreover, it was supposed that higher levels of apoptotic cells were the result of DNA damage caused by oxidative stress damage [42]. Epoxiconazole, an active agent of Tango®, was tested also. The results were very similar to those obtained in a commercial formulation; no clastogenic/aneugenic effects but a decrease in the CBPI were observed after 24/48 h treatment [47]. Other triazole fungicide formulations, namely a tebuconazole-based fungicide Orius® 25 EW and tebuconazole/prothioconazole-based fungicide Prosaro® were used in experiments; no significant increase in the MN frequencies but a significant decline in proliferation indices were observed [40, 46].

Results demonstrating the pesticide effect on micronucleus formation in sheep (*in vivo* or *in vitro*) are not so common. Tolylfluanid, a fungicide, was tested in sheep after 28-day chronic exposure [50] and in *in vitro* conditions [49]. In both cases, the fungicide was able to induce higher levels of MN. At the same time, no cytotoxic effect was seen. Then, the positive effect of vitamin E and selenium from the perspective of MN level reduction after carbon tetrachloride induction was observed in ovine lymphocytes [48].

Rabbits, as common small farm animals are also good bioindicators for genotoxicity assessment. Their advantages are: easy manipulation, sampling, housing and low cost compared to big animals. The effect of neonicotinoid imidacloprid was studied after oral administration for 2 to 4 months [44, 52]. Chronical exposure led to statistically significant MN induction with no changes in the CBPI. Similar results were found in rabbit lymphocytes after chronical treatment with cypermethrin, piperonylbutoxid and their mixture [53]. On the other hand, negative result in the ability to induce MN formation in bone marrow erythrocytes was obtained after bendiocarbamate administration after a 90-day chronical administration [36]. Simultaneously depression in bone marrow proliferation was seen.

Finally, the studies performed on poultry (chickens, ducks...etc.) were very rare. G i r i et al. [19] studied the effects of malathion on chicks. They detected significant MN elevation not only in the bone marrow (2.5, 5, 10 mg.kg^{-1}) but also in the peripheral cells (5 and 10 mg.kg^{-1}). Higher numbers of MN were also seen in broiler chicken blood erythrocytes after cypermethrin administration [43]. The MN reduction was seen after simultaneous treatment with vitamin E and selenium.

CONCLUSIONS

The micronucleus assays (*in vivo* or *in vitro*) together with chromosomal aberration tests are some of the officially approved tests by OECD for chromosomal damage testing. Our review informs briefly about the actual status and usage of MN assay in genotoxicity monitoring in aquatic environments and with farm animals. Testing the aquatic organisms is the best option of how to confirm or deny the presence of harmful chemical agents in the water and surrounding biotope because feed and water are the main sources of environmental exposure to farm animals. According to these results, we can conclude that the MN assay is a suitable method because of the simultaneous detection of aneugenic and clastogenic effects of chemicals. Moreover, in many cases a cytotoxic effect (reduction of CBPI) was observed even though there were no MN induction or vice versa. The use of CBMN cytome assay in peripheral lymphocytes provides an opportunity to study genotoxicity and cytotoxicity within *in vitro* and also *ex vivo*, which is important for modelling and predicting the *in vivo* genotoxic effects to chemical agents on humans.

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PREVALENCE OF PORCINE CIRCOVIRUS TYPE 2 IN NATURALLY INFECTED PIGS IN ABEOKUTA, NIGERIA

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ABSTRACT

Porcine circovirus type 2 (PCV2) associated diseases are a group of emerging devastating pig diseases worldwide. Due to a dearth of information on the virus in Nigeria, this study was carried out on 12 farms in Abeokuta Ogun State, Nigeria. Key production problems were identified through a questionnaire survey and direct field observations. The prevalence of the PCV2 was determined by the Polymerase Chain Reaction (PCR); 207 sera of pigs were obtained from the farms, DNAs extracted and amplified by the PCR. Based on gel results, the prevalence of PCV2 types were recorded and the effects of age, sex and geographic area determined. The observed production problems (and the percentage of farms suffering from such problems) were: runting (91.7%), high piglet mortality (83.3%), late term abortion (50%), stillbirth (50%) and anoestrous (41.7%). The overall prevalence of 8.7% of the PCV2 viral DNA was obtained. A single infection of PCV2b and PCV2a accounted for a prevalence of 5.3% and 2.4%, respectively, while a concurrent infection of both was found in only 1% of the pigs. The prevalence of PCV2 was not significantly ($P > 0.05$) influenced by age or sex; with the rates in adults, growers and piglets being 9.9%, 8.1% and 8.1%, respectively, while in males and females, the rates were 9.4% and 9.0%, respectively. The prevalence of PCV2 in high-density areas (13.2%) was significantly ($P < 0.05$) higher than in low-density areas (4.0%). It was concluded that PCV2a and PCV2b were present in pigs in Abeokuta, Nigeria and PCV2b was more prevalent. It was recommended that large-scale epidemiological studies covering all geographical regions be carried out with sequencing and phylogenetic analysis to characterize the PCV2 genotypes present in Nigeria.

Key words: Nigeria; PCR; PCV2 viral DNA; pig; Porcine Circovirus type 2; prevalence

INTRODUCTION

Porcine circovirus type 2 (PCV2) is the causative agent of porcine circovirus associated diseases (PCVADs), an

emerging disease complex devastating the pig industry worldwide. It consists of non-enveloped viruses that belong to the genus Circovirus, family Circoviridae. They contain a circular, single-stranded DNA genome of approximately 1.76 kb [24, 27]. Porcine circovirus type 2 and PCVADs are present on all continents and have been reported in many countries worldwide [4]. PCV2 persists as a subclinical infection in most commercial pig herds [33]. Both asymptomatic and diseased pigs shed virus or viral DNA in excretions and secretions [26], contaminating the premises, with the most susceptible being post-weaned pigs. The virus is stable and persists in the environment for prolonged periods of time, resulting in the exposure of young piglets. The mode of infection is widely believed to be through the oro-nasal route [34].

The group of diseases associated with PCV2 include: Post-weaning Multi-systemic Wasting Syndrome (PMWS), a multifactorial disease that usually appears in weaned pigs between 7 and 16 weeks of age [35]; Porcine Dermatitis and Nephropathy Syndrome (PDNS) is commonly seen in pigs that are 12—14 weeks of age [20]; Porcine Respiratory Disease Complex affects pigs from 8 to 26 weeks of age [17]; enteritis with PCV2 involvement often affects animals between 8 and 16 weeks of age [22]; reproductive failure affects mostly first parity gilts [38]; while congenital tremors affect newborn piglets [19].

Control and prevention strategies are two folds: improved management and biosecurity practices, and vaccination. The major goals of improved management and biosecurity practices include minimizing pig-to-pig contact, ideal biosecurity, improved hygiene and nutrition [25, 15]. Direct pig-to-pig contact has been identified as a potential means of viral spread in a herd [37]. Establishing solid partitions between pens and adopting all-in-all-out systems across the farm are recommended to reduce the pig-to-pig contacts [37]. Using only semen or boars of known health status for artificial insemination (AI) and breeding, prevents vertical transmission of the virus. The quarantine of newly purchased pigs and minimizing visitors to a facility helps to prevent the introduction of new infections. Thorough cleaning and disinfection procedures using an effective disinfectant such as potassium peroxomonosulphate, sodium hypochlorite, and sodium hydroxide [22], in addition to other hygiene measures including regular changing of boots and clothes, prompt removal of sick and dead pigs, proper disposal of dead pigs have been recommended to curb the spread of infection within herds/farms [37].

The prevalence of PCV2 viral antigen (or DNA) recorded in different countries varied widely, including: 23 % in Japan, 8 % in Korea, 35 % in the UK, 10 % in the USA, and 50 % in Taiwan [5]. In the sub-Saharan African regions, the virus has been detected and the disease reported only in three countries; South Africa [1, 12], Uganda [21, 28, 39] and Mozambique [23]. The prevalence of PCV2 DNA recorded also varied widely in these three countries, including: 15.93 % in South Africa [2], 77 % and 25 % in Uganda [21, 39], and 54 % in Mozambique, respectively [23].

PCV2 strains have been classified into four main genotypes (PCV2a, PCV2b, PCV2c, and PCV2d) based upon the phylogenetic analyses performed with their full genomes and ORF2 sequences [14, 40]. A fifth genotype, PCV2e has also been identified in Mexico and USA [16], all of which have neither been detected nor identified in Nigeria, although anti-PCV2 antibodies have been detected in pig sera at a prevalence of 1.4 % [3].

There is insufficient data on the prevalence and genotypes of PCV2 in the sub-Sahara African regions [1]. Particularly, there is a dearth of information on PCV2 in Nigeria and not much work has been done to detect its presence in the country. In this study, the prevalence of PCV2 was determined by the use of the Polymerase Chain Reaction (PCR).

MATERIALS AND METHODS

Study area, samples, data collection and the study animals

Targeted surveillance was accomplished in 12 selected pig farms in Abeokuta and environs, by multi stage simple random sampling. Abeokuta is positioned at 7°9'39'N and 3°20'54'E on the Ogun River. The four local government areas (LGAs) are Abeokuta South, Abeokuta North, Odeda and Obafemi-Owode. Abeokuta South LGA has a human population of 250,278 and an area of 71 km^2, Abeokuta North LGA has a population of 201,329 and an area of 808 km^2, Obafemi-Owode LGA has a population of 228,851 and an area of 1,410 km^2 while Odeda LGA has a population of 109,449 and an area of 1,560 km^2 [6].

A total of 207 blood samples were randomly collected from pigs with different age groups, irrespective of their health status. The pigs sampled were of mixed breeds (Nigerian indigenous breed, Large White, Duroc, Landrace,

Camborough) and were intensively managed. The age of the pigs sampled ranged from a day old to 24 months and were categorized according to the following production groups: 0—3months (piglets), 3—6 months (growers), and over six months (adults/breeders). The health status of each pig was recorded as sick or apparently healthy, based on the presence or absence of their clinical abnormalities. The clinical data were obtained by interviewing the farm owner, from farm records and/or by direct observation of the pigs and the farm environment. The sick pigs were physically examined and the clinical parameters noted. About 3 ml of blood was collected via the anterior *vena cava*. The serum was harvested by allowing the blood to clot undisturbed at room temperature. It was centrifuged at 1000 g for 10 minutes. The supernatant serum was pipetted and transferred into Eppendorf tubes and stored at –20 °C until used later.

Deoxyribonucleic acid (DNA) extraction

Deoxyribonucleic acid was extracted from the serum using Quick-gDNATMMiniPrep (Zymo Research Corporation, Irvine, CA 92614, USA) as described by the manufacturer. Briefly, 200 µl of genomic lysis buffer was added to 50 µl of serum in a sterile 1.5 ml Eppendorf tube, thoroughly mixed by vortexing for 4—6 seconds and incubated at room temperature for 5—10 minutes. The mixture of the serum and lysis buffer was transferred to a spin column in a collection tube and centrifuged at 10,000 g for 60 seconds, after which the collection tube with the flow through was discarded and the spin column transferred to a new collection tube. This was followed by adding 200 µl of prewashed buffer to the spin column and centrifuged at 10,000 g for 60 seconds, after which 500 µl of genomic DNA washed buffer was added to the spin column and centrifuged at 10,000 g for 60 seconds. The DNA was eluted by transferring the spin column into a new 1.5 ml Eppendorf tube and 50 µl of elution buffer was added to the spin column and incubated at room temperature. The eluted DNA in the Eppendorf tube was stored at –20 °C until used later.

Polymerase Chain Reaction

Two sets of species-specific primer sets (Table 1) were selected based on published data [41]. The sets of primers were optimized with DNA already extracted from serum samples positive for PCV2a and PCV2b obtained from the Research Centre for Animal Health (CReSA), Cerdanyola del Vallès, Spain, which were also used as positive controls.

Polymerase chain reaction amplification was performed according to [18, 41] in a 20 µl final reaction volume containing the equivalent of 20 ng of genomic DNA, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 µM KCl, 200 µM each of dNTPs, 40 ng of each of the primers and 1 unit of Thermusaquaticus DNA polymerase (Bioneer, USA). Both sets of primers were used to perform PCR for each field sample separately, including the positive control samples. The mixtures were placed in a My Cycler (BIORAD, USA) with the following reaction conditions:

Polymerase chain reaction for PCV2a, and PCV2b involved an initial denaturation at 94 °C for 5 min, followed by 40 cycles at 94 °C for 15 s, 60 °C for 20 s, 72 °C for 30 s, and a final extension for 10 min at 72 °C. The PCR reaction products were visualized by electrophoresis on a 1.5 % agarose gel.

Table 1. Primer sets names, sequences and sizes of the expected amplicon

Primer set	Species	Sequences (5′–3′)	Expected band sizes	References
FPCV2ab				
RPCV2a	PCV2a and PCV2e	CAGTTCGTCACCCTTTCCC	546bp	(Z h a i et al., 2011)
		GGGGGACCAACAAAATCTC		
FPCV2ab				
RPCV2b	PCV2b, PCV2c and PCV2d	CAGTTCGTCACCCTTTCCC	547bp	(Z h a i et al., 2011)
		GGGCTCAAACCCCCKCWC		

Statistical analysis

The raw data were entered into a Microsoft Excel spreadsheet and descriptive statistics was used to present the data. The Statistical Package for Social Sciences (SPSS) version 24.0 was used for the data analysis. The prevalence of the infections within age groups, sexes, and farm locations were compared using the Chi-square test. A $P < 0.05$ was considered significant.

RESULTS

The band sizes of 546 bp and 547 bp corresponding to the expected band sizes of PCV2a and PCV2b respectively, were obtained on the agarose gel after electrophoresis (Figures 1 and 2). The prevalence of PCV2 in naturally infected pigs by the PCR in relation to virus genotype is presented in Table 2. The viral DNAs were detected in 8.7 % of all of the samples. The prevalence of the PCV2 in relation to the pig age group, sex and geographic area are presented in Table 3. There were no significant ($P > 0.05$) differences in the prevalence of PCV2 among the pig age groups. There was also, no significant ($P > 0.05$) differences in the prevalence of PCV2 between the sexes. There was a significant ($P < 0.05$) difference in the prevalence of PCV2 between the high-density and low-density areas. The farmer's opinions on the presence of clinical signs associated with the PCVADs on their farms were said to include: runting, high piglet mortality, late term abortion, still birth and anoestrous in gilts and sows (Table 5). The piglets and growers sampled were observed to show slow growth and emaciation (Figure 3).

Table 2. Prevalence of PCV2 in naturally infected pigs by PCR in relation to virus genotype

Genotype	No. positive out of 207 samples	Prevalence [%]
PCV2a	5	2.4
PCV2b	11	5.3
Concurrent PCV2a and PCV2b	2	1.0
Total No. positive	**18**	**8.7**

DISCUSSION

A previous study on the prevalence of PCV2 conducted in Ibadan, Nigeria recorded a prevalence of 1.4 %, based on screening for IgG antibodies against PCV2 [3]. Hence, our study in Abeokuta complements the previous study and provides information on the detection and the prevalence of PCV2 viral DNA, which indicates that both PC-

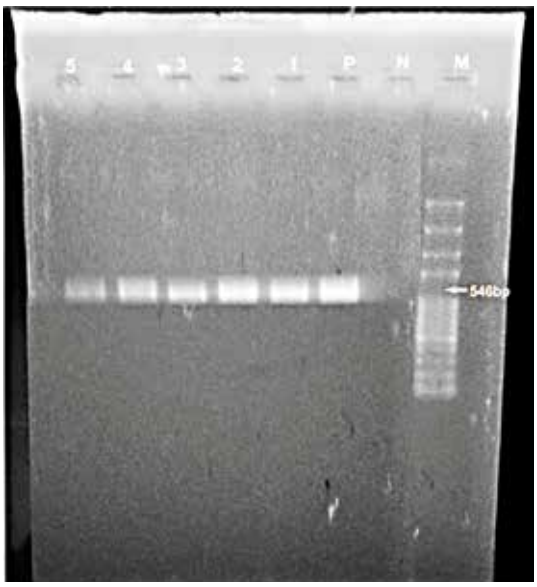

Fig. 1. Agarose gel with the products of PCR amplification of genotype PCV2a from serum samples
Lane M—Molecular weight standards (100 bp ladder); Lane P—Positive control; Lane N—Negative control; Lanes 1—5 are positive field samples

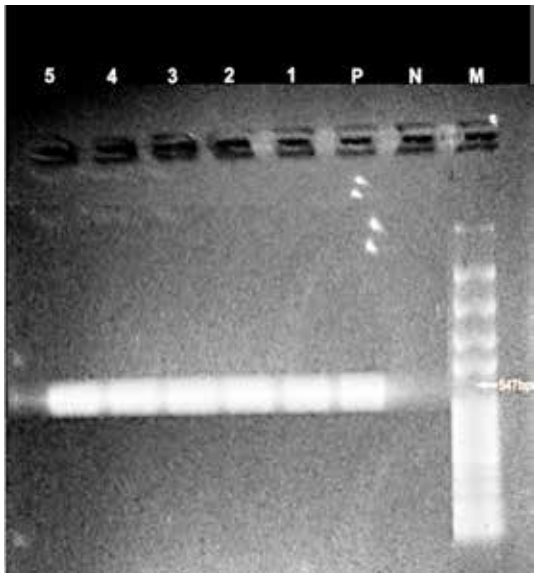

Fig. 2. Agarose gel with the products of PCR amplification of genotype PCV2b from serum samples
Lane M—Molecular weight standards (100 bp ladder); Lane P—Positive control; Lane N—Negative control; Lanes 1—5 are positive field samples

Table 3. Prevalence of PCV2 in relation to pig age group, sex and geographic area

	Number sampled	Number positive for PCV2	Prevalence [%]	P value	Sign.
		Age group			
Piglets (0—12 weeks)	74	6	8.1		
Growers (13—24 weeks)	62	5	8.1		
Adults (> 24 weeks)	71	7	9.9		
Total	**207**	**18**	**8.7**	**0.184**	**0.912**
		Sex			
Male	85	6	9.4		
Female	122	12	9.0		
Total	**207**	**18**	**8.7**	**0.612**	**0.434**
		Geographic area			
aHigh density areas	106	14	13.2		
bLow density areas	101	4	4.0		
Total	**207**	**18**	**8.7**	**5.57**	**0.018***

aHigh density area (Abeokuta North & Abeokuta South LGAs), P < 0.05;
bLow density areas (Obafemi-Owode & Odeda LGAs); *—significant

Table 4. Farmers' opinin on the presence of clinical signs associated with PCVAD on their farms

Clinical signs of PCVAD	Number of farmers With problems	Percentage [%] of all (12) farms
Runting	11	91.7
High piglet mortality	10	83.3
Late term abortion	6	50.0
Stillbirth	6	50.0
Anoestrus in gilts/sow	5	41.7

Fig. 3. Sick piglets in one of the sampled farms showing growth retardation and emaciation indicated by downward and upward arrows respectively

V2a and PCV2b are present in pigs in Nigeria. Our study revealed an overall prevalence of 8.7 % for PCV2 DNA in the serum of pigs by the PCR method. This is comparable to the prevalence of PCV2 DNA reported for Korea and USA [5]. Higher PCV2 prevalence has also been reported for South Africa [2], Uganda [39], Slovakia [11], Colombia [32], Japan and the UK [5]. Also, much higher prevalences has been found in Uganda [21] Mozambique [23] and in Taiwan [7] by the PCR.

Many factors could be responsible for the variations reported in the prevalence across countries, such as the period of sample collection. Studies have revealed that samples collected during disease outbreaks, or thereafter gave a higher prevalence than during the periods of no disease outbreak. In Taiwan, a prevalence of 50.0 % for PCV2 DNA was recorded during the first PCV2 disease outbreak [7]. Thereafter, a much higher prevalence of PCV2 infection in pigs and pig herds (68.8 %) and (92 %), respectively were

observed [8]. The samples employed in this study were collected during periods of no report of any disease outbreak. The absence of an overt disease outbreak may thus explain the low prevalence obtained in this work. Another possible reason for variation in the prevalence is the production group of pigs the study focused on. Our study cut across all production groups of pigs. On the contrary, a study that focused on grower/finisher group in the USA reported a PCV2 high prevalence of over 82.0 % [31]. This suggests that focusing on a particular production system may influence the disease prevalence.

The choice of the sets of primers used in this study was selected by the possibility of detecting other PCV2 genotypes and multiple strain of co-infections. However, attempts to obtain positive DNA samples that would serve as positive controls for the other genotypes were not successful; therefore, this work was focused on detecting only PCV2a and PCV2b. This can however, be confirmed by sequencing and phylogenetic analysis, which, were not included in the objective of our study. Thus, our present study has provided a good foundation for further studies on the molecular characterization of PCV2 in Nigeria. The higher prevalence of PCV2b than PCV2a recorded could be due to a genotypic switch from PCV2a to PCV2b overtime. This is supported by other published reports [9, 10, 13, 30]. The concurrent infection of PCV2a and PCV2b obtained is an indication that more than one genotype can possibly co-infect the same pig under natural infection. This is also consistent with some earlier reports [18, 41].

Our studies revealed that the pig farmers were of the opinion that runting, high piglet mortality, late term abortion, still birth and anoestrous in gilts and sows, which are problems associated with PCVAD, were present in their farms and are also the most common factors limiting the productivity of their pigs. Out of 136 growers studied, 52.9 % were runted while 36.8 % were emaciated. All of the three farms examined in Abeokuta South LGA were positive for PCV2 infection, whereas two farms out of three examined in each of the other three LGAs namely; Abeokuta North, Obafemi-Owode and Odeda were positive for PCV2 infections. It was observed that the three farms where PCV2 infection was not detected had a reasonable level of farm management and biosecurity measures in place. They had foot dips containing disinfectants at the farm entrance, restricted movement of people were practiced, and workers wore protective clothing, boots, and gloves during the farm operations. Isolation and quarantine facilities were also available. These farms also practiced the all-in-all-out system and close weight range of piglets on entry to nursing. This may explain why, in contrast to other nine farms, PCV2 prevalence was 0 % on these three farms. These findings agree with several epidemiological studies, which have shown that management, housing and husbandry factors were strongly related to the risk of developing PCVAD [33]. Rodent control was absent in all the farms thus predisposing to infections, especially in those farms with poor hygiene. This is an issue of concern, as rodents are possible risk factors in the transmission of PCV2 [29].

It was observed in our study, that adult pigs (gilts, sows and boars) had a slightly higher rate of infection than the piglets (nursery and weaners), and the growers (growers and finishers). Even though there is variation in clinical forms of the disease across age groups, there was no significant difference between the occurrence of PCV2 infection and age groups in this study. This agrees with an earlier report that compared the amount of PCV2 DNA in PCVAD cases and did not identify any age-related association [36].

The prevalence of PCV2 in males was not significantly higher than in females, therefore suggesting that the infection of PCV2 was not influenced by sex. The higher prevalence in Abeokuta South LGA and Abeokuta North LGA than Obafemi-Owode and Odeda LGAs may be due to the higher stocking density of pigs due to space constraints in the urban areas. In addition, most of the pig farms in the high-density areas were in close proximity to each other. Obafemi-Owode and Odeda LGAs that are more sparsely populated, had much lower prevalences. The pig farms in these LGAs were located far away from residential areas and from each other, thereby possibly reducing the risk of PCV2 transmission in these areas. The breed prevalence of PCV2 infection was not studied because all of the pigs sampled were crosses of various breeds.

Although there were no pigs with a definitive diagnosis of PCVADs in this study, four on-farm observations suggested that some farms might have had an outbreak of the disease at some point in time: firstly, weak management practices including inadequate housing facilities and biosecurity measures; secondly, three farms had a recent history of unusually high rates of piglet mortality, infertility, abortion and still births, and large numbers of runts in the

piglet and grower groups; thirdly, the owner of farm 6 reported that the pigs on the farm did not respond to mass treatment, following a presumptive diagnosis of trypanosomiasis, a disease with similar clinical signs as PCVADs in pigs; and fourthly, both single and mixed infections of PCV2 DNAs were detected in both apparently healthy and the sick pigs on the farms. The scope of our work did not cover the diagnosis of PCVADs. In light of the findings in this study however, such investigations are required as PCVADs may be a differential diagnosis of trypanosmiasis in pigs in the study areas.

CONCLUSIONS

This work reports on the detection of PCV2 DNA in serum samples of pig populations in Abeokuta, Nigeria, even though the clinical disease has not been diagnosed in the country. It is therefore recommended that large-scale epidemiological studies covering all geographical regions be carried out to diagnose and determine the overall prevalence of PCV2 and PCV2 genotypes in the country by sequencing and phylogenetic analysis.

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PROTECTIVE EFFECT OF FLAXSEED ON THE HEALTH OF EXPERIMENTAL ANIMALS EXPOSED TO XYLENE

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ABSTRACT

Xylene is mainly used as a solvent in the printing, tire and leather industries. It is also used as: a facility cleaner, paint and varnish thinner, component of fuel, and chemical for the laboratory processing of histological preparations. For these reasons people are frequently exposed to xylene and the risk of intoxication is high. This study focused on the protective effect of flaxseed on mice experimentally intoxicated with xylene. The experiment lasted 14 days. The mice used in this study (n = 60) were allocated to 3 groups: the control group (C) received only the standard diet; the xylene group (X) was fed a standard diet and was administered xylene p. o. (10 µl daily); and the xylene + flaxseed group (XF) received the standard feed, crushed flaxseed and xylene at the same dose as group X. The observations involved changes in: body weight, liver enzyme levels, and caspase activity in the liver of the mice. The administration of additives resulted in significant changes in the body weight of the mice on day 7 of the experiment ($P < 0.05$). The highest weight gain was observed in mice from the XF group. In contrast, the body weight of the mice from group X exposed only to xylene was the lowest. The biochemical analysis of the liver cells of the xylene intoxicated mice showed elevated levels of: aspartate aminotransferase (AST), De Ritis ratio (AST/ALT ratio), and lactate dehydrogenase isoenzymes LDH-3 and LDH-5. Caspase-3, the marker of apoptosis, was increased in the XF group. Thus, the administration of flaxseed in our experiment had a beneficial effect on the clinical and metabolic parameters of mice intoxicated with xylene. Our results indicated that the administration of flaxseed, may act as a preventative measure with respect to xylene intoxication of animals; however, further analyses are needed to confirm this assumption.

Key words: crude oil; flaxseed; lactate dehydrogenase; mouse; xylene

INTRODUCTION

Crude oil is used in various branches of industry. Together with its side products it is used for: production of

fuel, plastic materials, acrylates, dyes, cleaning preparations, textile fibres, pesticides, and other auxiliary compounds intended for industrial use. Crude oil extraction presents also considerable environmental risks [20]. It contributes to air pollution, acid rains, damage to the ozone layer, and the development of various diseases in humans and other animals. Currently, many scientists point to the absence of studies leading to an accurate definition of the harmful effects of crude oil and its intermediate products, for example xylene, on humans or other animals living close to oil wells. Xylene is a chemical the scientists and laboratory workers are frequently exposed to during the processing of some laboratory samples [6]. It is necessary to improve the control of many contaminants in the environment, for example in air, water and soil and focus on the prevention of their harmful effects.

Current research focuses on the utilization of biologically active additives of natural origin that may protect or improve the health of consumers. Such additives include also flaxseed. The curative effects of common flax (*Linum usitatissimum*) have been described in studies that investigated the development of: gastrointestinal diseases, prevention of diabetes, cardiovascular diseases, atherosclerosis and hypertension [34]. Flax is a rich source of n-3 polyunsaturated fatty acids (PUFA), particularly the essential α-linolenic acid (ALA). The n-6 and n-3 PUFA are an integral parts of cell membranes and are responsible for their fluidity and other physicochemical properties. K o m p r d a [23] reported that for the maintenance of good health of humans, the n-6 : n-3 ratio in their nutrition should be as close as possible to 1 : 1. In the advanced countries the food generally contains more n-6 and very little of n-3 PUFAs which is considered as one of the reasons for the high prevalence of chronic and degenerative diseases [7]. Enzymatic oxidation of n-3 PUFAs produces eicosanoids which act as tissue hormones participating in reproductive, immune, secretory and growth processes. In the intestine, under the action of microorganisms, the processes of dihydroxylation, dehydrogenation and demethylation transform lignans to enterolacton and enterodiol that pass through the intestinal wall and are absorbed into the blood and tissues [14, 38]. The chemical structure of these compounds resembles that of 17β-oestradiol. They bind to oestrogen receptors of target cells and support cellular proliferation and apoptosis [32] and some other factors affecting the cellular cycle [29, 41]. Under both *in vitro* [21] and *in vivo* [36] conditions, flax promotes apoptosis of somatic cells of ovaries and oocytes in pigs. Some studies have confirmed the pro-apoptotic effect of flax related to the high content of antioxidants, such as flavonoids [17]. Another effect of n-3 PUFA is related to their action at the level of cell membranes involving the regulation of the flow of potassium ions into the cells (cervonic acid) [26]. One of the ways of evaluation of the integrity of cell membranes is the determination of the activity of lactate dehydrogenase (LDH), an enzyme found in all organs and tissues including animal blood [4, 22]. Its increased or decreased release into the environment is considered a valuable marker of the integrity and viability of cells [25].

The aim of this study was to obtain new knowledge about the influence of flaxseed on mice experimentally intoxicated with xylene under *in vivo* conditions.

MATERIALS AND METHODS

Experimental animals and groups

This study was carried out using 60 female mice of line CD-1 ICR (Velaz, Czechia). Before the onset of the experiment, at the age of 28 days, these mice were transferred from the breeding establishment to the quarantine section of the experimental facility of the Institute of Physiology of Farm Animals of the Slovak Academy of Science in Košice. After reaching the age of 35 days, they were divided to three groups with 20 mice in each: group 1 (control—C) the mice were fed the standard commercial feed (M3; BONAGRO a.s. Blažovice CZ, Czechia; Table 1) at a dose of 4 g per day (in two feedings); group 2 (xylene—X) was fed the standard diet and administered xylene p. o. (10 μl.day^{-1} by cannula); and group 3 (xylene and flaxseed XF) was fed the standard diet supplemented with crushed flaxseed (var. Libra, 10 % concentration in the feed) and administered xylene at the same dose as group 2. The experiment lasted 14 days. The mice were kept in boxes (6—7 mice in each) on bedding (Lignocel 3-4S), at a temperature of 20—24 °C, relative humidity of 45—65 % and a 12-hour light regimen. Their weight was regularly recorded. After 14 days, they were sacrificed by cervical dislocation (Experiment approval No. 598/18-221/3) and their livers were collected for analysis of: alanine transferase (ALT), aspartate aminotransferase (AST), activity of lactate dehydrogenase (LDH) and its isoenzymes and the activity of caspase.

Table 1. Composition of the standard diet M3

Crude protein	22 %	**Vitamin A**	20 000 IU
Crude fibre	3.14 %	**Ferrous sulphate monohydrate**	17 000 mg
Coarse fat	3.31 %	**Potassium iodide**	0.65 mg
Ash	6.16 %	**Cobalt (bis)carbonate**	0.4 mg
Calcium	1.01 %	**Copper sulphate pentahydrate**	15 000 mg
Phosphorus	0.53 %	**Manganese oxide**	45 000 mg
Sodium	0.11 %	**Zinc oxide**	71 000 mg
Vitamin D3	2 000 IU	**Selenium**	0.15 mg

Spectroscopic analyses

The spectroscopic analyses were carried out employing a spectrophotometer ALIZÉ (LISABIO, Pouilly-en-Auxois, France). We applied 30 µl of a sample and made it up to 500 µl with a substrate. This method was used to determine: the total proteins (necessary for recalculation of the specific activity of enzymes), the concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and the activity of the total lactate dehydrogenase (TLDH).

Electrophoretic analysis of lactate dehydrogenase isoenzymes

The LDH isoenzymes were determined in the liver extracts. The samples were processed using an automatic, multiparametric, electrophoretic system HYDRASYS (SEBIA, Lisses, France). Dry gels were evaluated visually and densitometrically (λ 570 nm; EPSON PERFECTION V 700 PHOTO) and the individual fractions were quantified using a software PHORESIS (Version 5.50, 2009, SEBIA, Lisses, France).

Caspase activity

The western blot analysis was performed using the supernatants of the liver extracts prepared for electrophoresis. We used 5 % defatted milk in TBST (1M Tris-HCl s pH 7.5, 1M NaCl, 0.01% Tween 20) to block the non-specific binding of the antibodies. After the migration of proteins in a polyacrylamide gel, we applied primary monoclonal antibodies (anti-caspase 3 antibody) using a 1 : 1000 dilution (Santa Cruz Biotechnology Inc., Dallas, USA) and incubated the gels overnight at 4 °C. On the second day, we applied secondary anti-mouse IgG antibodies using 1 : 20 000 dilution (Sigma-Aldrich). The β-actin antibodies diluted 1 : 10 000 (Thermo-Fisher) were applied as a control. The reaction on the PVDF membrane was visualised by means of a chemiluminescence kit (Clarity™ Western ECL Blotting Substrate, Biorad) and scanned by an instrument Fusion-Fx7-Spectra (Vilber Lourmat, Eberhardzell, Germany).

Statistical processing of the results

The results obtained in our study were processed by a software GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, California, USA). An unpaired t-test was used to determine the significance of differences between groups at the end of the experiment. Our results were presented as arithmetic means and the standard errors of means (Mean ± SEM). We found significant differences at the following levels: $P < 0.05$; $P < 0.01$; $P < 0.001$.

RESULTS AND DISCUSSION

Due to the intoxication with side products of the crude oil industry, the prevalence of various diseases of humans and other animals has increased recently. One of the potential preventive measures involves the use of natural additives including the common flax. This plant has beneficial effects on the health of individuals. Common flax was put to use in people with gastrointestinal problems, for example with constipation, colitis or enteritis, owing to its high content of fibres and other components [8]. Flaxseed has also been used in the treatment of cardiovascular dis-

eases, atherosclerosis and hypertension [1, 12]. It supports the treatment of acne and diabetes, it is used to alleviate the symptoms of menopause and helps to manage obesity. Beneficial effects of flax include the prevention of cancer of the mammary gland, prostate, lungs and the colon [9, 10, 37, 39].

Our experiment involved 14-day observation of mice allocated to three groups subjected to different treatments. The body weight of mice fed the standard diet and exposed to xylene p. o. (X) was reduced the most throughout the experiment (Fig. 1). Similar results were reported in the toxicological review by United States Environmental Protection Agency (US EPA) in which the mice intoxicated with o-xylene showed a reduction in weight gain by about 15 % during 6 or 12 months of administration of xylene [35]. By day 7 of our experiment, a significantly higher body weight ($P < 0.05$) compared to group X was observed in mice from group XF, the diet that was supplemented with flaxseed (10 % concentration in the feed) and also exposed to xylene (Fig. 1). The increased body weight after supplementation of flax was also observed by Juárez et al. [19]. On the other hand, Mohammadi-Sartang et al. [30] and Pourjafari et al. [31] recorded decreased body weight in humans who consumed flaxseed at doses ≥ 30 g for ≥ 12 weeks.

The increased serum levels of the amino transferases ALT and AST indicated an acute or chronic damage to the liver. The increased AST also suggested a non-specific damage to tissues including: the liver, brain, pancreas, heart, kidneys, lungs and skeletal muscles. The AST levels determined in the liver cells of mice from group X were significantly higher ($P < 0.05$) in comparison with the group XF supplemented with flaxseed (Fig. 1). This indicated some damage to the liver parenchyma of the mice. The increased activities of AST and ALT after the inhalation exposure to xylene were recorded by Kükner et al. [24]. Similar to our experiment, the positive effects of flaxseed supplementation of the diet toward normalizing the levels of AST and ALT enzymes were described in the studies by Al-Bishri [2] and Zhang et al. [42].

The De Ritis ratio (serum AST/ALT ratio) indicated the degree of necrotic or other damage to the liver cells. The higher the coefficient, the more severe damage to the liver parenchyma is expected. In people this value must not exceed 1.0. The increase in this parameter indicated various pathological changes: AST/ALT ≤ 1 indicated viral hepatitis, AST/ALT > 2 cirrhosis (any aetiology); and AST/ALT > 4 acute liver failure [28]. The maximum in mice is 1.38 [13]. In our experiment, this coefficient was increased in group X (Fig. 1) which indicated the hepatotoxic effect of xylene. In the control C and XF groups, this parameter was in the physiological range (< 1.38) which suggested the hepatoprotective effect of flaxseed.

The determination of the activity of lactate dehydrogenase in the serum has a broad clinical significance. Changes in the activity of LDH in cells and tissues have been considered a valuable marker of damage to cellular membranes, for example by xylene. We determined the activity of LDH in the extracts of liver homogenates. The LDH is an intracellular enzyme that after release to the surrounding environment indicates damage to tissues [4, 5, 22, 40]. The activity of the total lactate dehydrogenase in the liver sample extracts was higher in groups X and XF in comparison to the control group C, although the difference was insignificant (Fig. 2). The ability of flax oil to decrease the activity of serum LDH was observed in the study by Hussein et al. [18]. Polyunsaturated fatty acids originating from flax are capable of incorporating in cellular membranes and affecting their fluidity and other properties by means of the modulation of the phospholipid bilayer and changes in micro-domains that act as signal platforms regulating the transport of cholesterol, transduction of signals and endocytosis [27]. The increased intake of n-3 PUFAs can decrease the level of cholesterol in the body [16, 33] and its content in cellular membranes which can result in an increased incorporation of n-3 PUFAs and changes in the composition of membrane phospholipides [16].

The determination of the dominant isoenzyme allows one to identify the damaged organ. The isoenzyme LDH-1 is found particularly in the heart, erythrocytes and brain. Isoenzyme LDH-2 is present in leucocytes and in lower proportion than LDH-1 also in erythrocytes. The LDH-3 isoform is typically associated with the lung tissue. Isoenzyme LDH-4 has been detected in kidneys, pancreas, and lymphatic nodules; and LDH-5 presence is characteristic of the liver and skeletal muscles [15]. Our study showed significant differences in the levels of isoenzymes LDH-3 and LDH-5 ($P < 0.05$), and the LDH-5 isoenzyme was the one that indicated damage to the liver in the group exposed to xylene. On the other hand, the group XF that was fed also flaxseed showed a decrease in the concentration of these isoenzymes in comparison with group X which in-

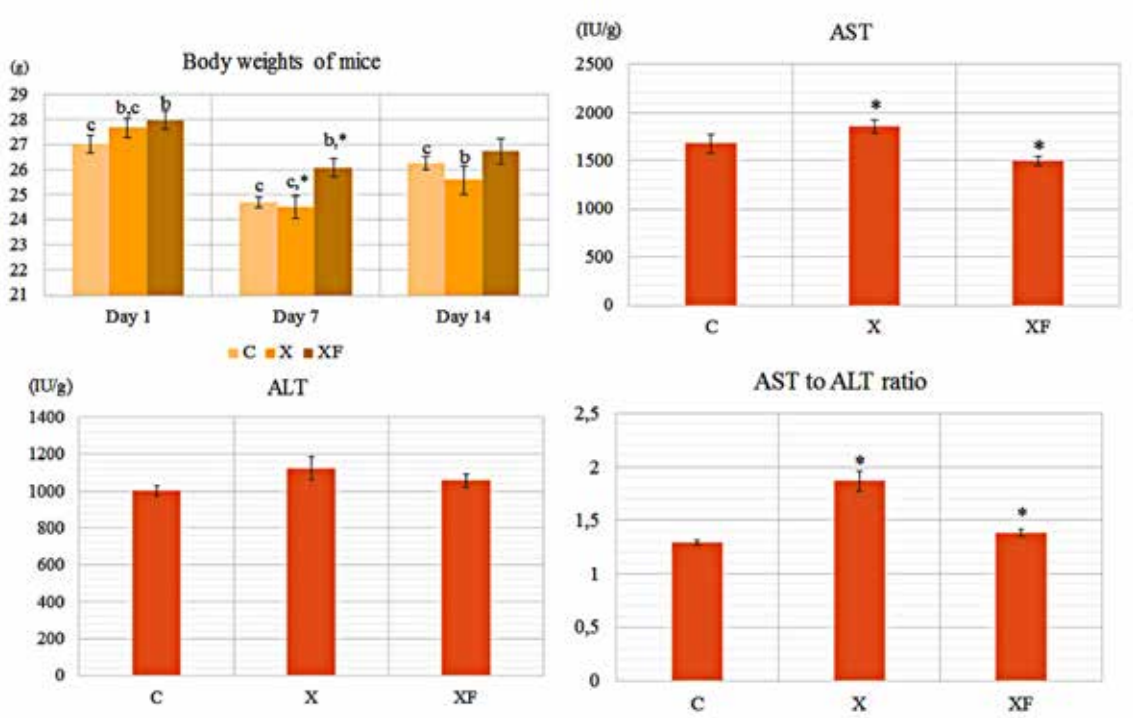

Fig. 1. Body weight changes, activity of AST and ALT enzymes and their ratio in control and experimentally intoxicated mice

C—control; X—xylene; XF—xylene and flaxseed. The results (mean ± SEM) marked with the same superscripts b, c,* are statistically significant; superscripts b,c express dynamics of changes within the group and asterix * expresses significance of differences between the groups at the end of the experiment: * = P < 0.05; b = P < 0.01; c = P < 0.001

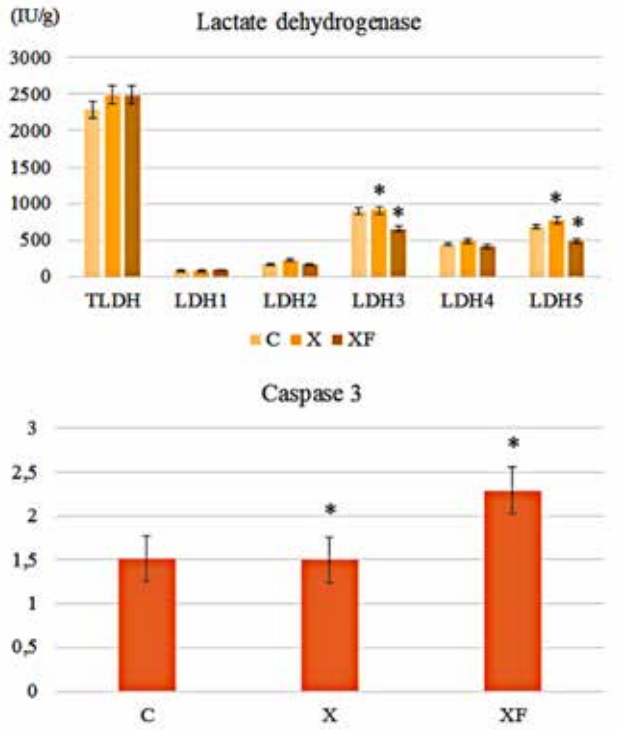

Fig. 2. Activity of total lactate dehydrogenase and its isoenzymes and intensity of immunoreaction (optical density) of caspase 3 in the liver of mice

C—control; X—xylene; XF—xylene and flaxseed. The results (Mean ± SEM) marked with asterisk differed significantly (P < 0.05)

dicated a hepatoprotective effect of flaxseed supplementation. These results agree with those presented in the study by A n d r e j č á k o v á et al. [5] in which the diet supplemented with flaxseed affected the release of the same isoenzymes (LDH-3 and LDH-5).

Our study included the observation of caspase 3 as a marker of apoptotic changes in the liver of intoxicated mice. Apoptosis is the main mechanism for the elimination of useless cells during development and maintenance of homeostasis in the healthy tissue. Thus, dysfunction of the apoptotic system results in pathogenesis of various diseases including cancer [17]. Our experiment revealed an increased expression of caspase 3 in group XF (Fig. 2), i. e. the group fed the diet fortified with flaxseed, in comparison with group X (P < 0.05) or the control. These results indicated the pro-apoptotic effect of supplementation of the diet with flaxseed on intoxicated hepatic cells. Similar results were obtained in the study conducted by A l - G h a m d i et al. [3] who reported that the long-term exposure to organic solvents xylene or toluene was associated with apoptosis of cells in the proximal renal tubules that could lead to their failure. Our observations of the

intoxicated mice, support the theory that the flaxseed oil may cause specific inhibition of the growth of tumorous cells and induce apoptosis of some tumour cells and thus exhibit anti-cancerous therapeutic potential [11].

CONCLUSIONS

The supplementation of diets with flaxseed of mice experimentally intoxicated with xylene resulted in a positive influence on their weight gain and selected biochemical parameters. We recorded a gradual increase in the body weight of mice that were fed flaxseed in comparison with the control group of animals. Thee supplementation of flaxseed alleviated the effects of intoxication which was reflected in the decreased activity of: the AST enzyme, De Ritis index, concentration of LDH and its isoenzymes LDH-3 and LDH-5 and of caspase 3 as a marker of apoptotic changes.

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INCIDENCE AND MORTALITY FROM A NEGLECTED TROPICAL DISEASE (RABIES) IN 28 AFRICAN COUNTRIES

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ABSTRACT

Rabies, a zoonotic disease, is one of the deadliest and most serious threats to public health as it has an almost 100 % case fatality rate. The global estimated mortality of the virus is between 40,000 to 70,000 deaths annually with most of the death occurring in the developing countries of Africa and Asia. The objective of this study was to present the incidence and mortality rates from rabies in 28 African countries from 2005 to 2018. Secondary data were obtained from the World Organization for Animal Health Database. The data from 2005 to 2018 were used, as this was the period with available data in the database. The data were analysed using SPSS version 25 and other descriptive statistical tools. The highest combined rabies incidence and mortality in the time range (2005—2018) was 1601 in 2006, while the lowest was 157 in 2005. Just five countries (Angola, Central African Republic, Kenya, Mozambique and Senegal) had 65 % of the rabies cases and mortality. Notably, the data on the incidence and mortality were 100 % similar, as all of the cases of rabies in the 28 African countries within 2005—2018 resulted in death. Therefore, more work should be devoted to research on rabies prevention and cure. Toward that goal, practices and policies should be implemented to enable the acquisition of accurate and consistent rabies data.

Key words: Africa; incidence; mortality; Neglected Tropical Diseases; rabies

INTRODUCTION

Rabies being a zoonotic disease, makes it one of the most deadly and serious threats to public health with an almost 100 % case fatality rate [5]. This virus infects domestic and wild animals; with dogs being the most significant reservoirs and they pose the greatest risk to humans. The infection is passed from animals to animals or from animals to humans through bites, scratches or mucous membrane exposure from these animals. The global estimated mortality of the virus is between 40,000 to 70,000 deaths annually with most of the deaths occurring in the develop-

ing countries of Africa and Asia [1]. The annual preventable deaths in these countries occurs where animal control, vaccination programs and post exposure prophylaxis are not properly implemented [14].

The genus Lyssavirus is the causal agent of rabies infection. It is a single stranded, negative-sense RNA virus that belongs to the family *Rhabdoviridae*. The virus replicates locally at the site of injury and gains entry to the central nervous system (CNS) [7, 22]. At these sites a productive infection ensues and the virus travels outwards to innervated organs; most notably the salivary gland. As a result, bite injuries are the most common first step in the acquiring of human rabies infection [14]. The domestic dog is an important vector in the transmission of human rabies that contributes about 97 % of all rabies related deaths in humans worldwide [25]. The disease is endemic in the rural poor communities where measures to prevent dog to human transmission have not been properly implemented [10]. Safety measures such as wound cleansing within a few hours after bites from suspected rabid animals may inhibit the onset of rabies and death. An average of 15 million people worldwide receives post exposure preventive regimen for rabies at an annual cost of $ 583.5 million with most of the beneficiaries living in developed countries [13]. Therefore, the most cost-effective approach to the elimination of the global burden of human rabies is by controlling canine rabies rather than the expansion of prophylaxis for human control. The resultant effect of mass vaccination campaigns of dogs and the control of the canine population has led to the elimination of rabies in terrestrial carnivores in several countries worldwide [14].

The regions that are mostly affected by rabies virus are the tropical countries in Africa, Asia, South America and Oceania. In Africa, rabies is considered as under reported, due to the absence of reliable surveillance data. Active surveillance studies have shown the difference between "officially recorded" and "likely occurring" rabies deaths [14]. Also, death following the onset of the clinical signs of the disease makes it difficult for most rabies cases to be reported or diagnosed in health facilities. In addition, the misdiagnosis of other neurological infections specifically in malaria endemic areas are common [12]. The dearth of life-saving post-exposure prophylaxis (PEP) and centres that provide PEP [18] for bite victims, as well as poor monitoring sales of PEP to private suppliers, all contributes in complicating the collection of appropriate data. Therefore, in the absence of reliable active wide spread surveillance studies, extrapolations are relied on to estimate the global burden of rabies [2].

The objective of this study was to make the best possible estimate of the burden of rabies in Africa, following its trend in the various African countries by combining and analysing all available data that allows us to estimate missing components. We built upon earlier data that allowed us access the current status of canine rabies in Africa and make available country specific assessments of the disease burden. This information is essential in planning effective control strategies.

MATERIALS AND METHODS

This study involved the use of secondary data on rabies incidence and mortality from the countries with available data on the World Organization for Animal Health Database [24]. The authors had to compile the data on an excel sheet as data on a specific indicator, year and country so that they could be compared. The data from 2005 to 2018 were used as this was the period with available data in the database. The descriptive statistics of frequencies, mean and standard deviations were used in analysing the data. Also, the data were analysed using the Statistical Package for Social Sciences version 25. No ethical approval was obtained for the study as this was not applicable.

RESULTS

The data were obtained for 40 African countries but only 28 could be used as the remaining 12 had very sparse data. Table 1 shows more details about the incidence data in years 2005, 2010, 2015 and 2018 and the mean values for the data from 2005 to 2018.

The highest combined rabies incidence in the time range (2005—2018) was 1601 in 2006, while the lowest was 157 in 2005. Mozambique was responsible for almost all cases (92.9 %) in the year with the highest rabies cases (2006). Also, Kenya had the highest number of rabies cases throughout the time period (2005—2018), i. e. 2262. Just five countries (Angola, Central African Republic, Kenya, Mozambique and Senegal) had 65 % of all rabies cases. One of the most drastic changes in the rabies incidence

Table 1. Rabies incidence and mortality in 28 African countries

Countries	2005	2010	2015	2018	X (Mean)*
Algeria	31	9	20	–	19.45
Angola	–	151	69	–	170
Benin	–	–	5	–	7.67
Burkina Faso	–	–	8	–	14.3
Burundi	–	–	22	–	15
Central African Republic	–	–	–	–	436.33
Congo (Dem. Rep. of the)	–	–	27	–	36.25
Cote D'Ivoire	–	2	–	25	9.56
Egypt	–	–	55	–	57.75
Eritrea	–	–	10	13	13.5
Ethiopia	–	28	–	–	197.67
Ghana	1	19	25	–	13.75
Guinea-Bissau	–	–	9	–	4.29
Kenya	–	3	–	744	226.2
Lesotho	9	–	4	–	64.63
Mali	–	18	7	2	31.25
Malawi	8	–	3	–	31
Morocco	25	19	19	18	20.38
Mozambique	43	24	29	69	155.36
Namibia	30	13	23	1	14.9
Nigeria	–	–	120	11	106.2
Senegal	–	2	2	1	105.73
South Africa	7	16	8	16	11.36
Sudan	–	9	–	–	5.67
Tanzania	–	–	45	93	54.8
Togo	–	17	1	–	12.18
Tunisia	3	–	6	3	3.33
Zimbabwe	–	–	2	14	11

– —No available data
*—Mean value is for the data from 2005 to 2018 and not for the years presented in the table

occurred in Mozambique as it increased from 43 in 2005 to 487 in 2006 and 53 in 2007.

Notably, Kenya's rabies incidence and mortality has been greater than 700 every year since 2016. Guinea-Bissau, Tunisia and Zimbabwe had a fairly low constant rabies incidence and mortality with the highest number of cases less than 20. Table 1 shows the data on mortality in the 28 African countries in some selected years (2005, 2010, 2015 and 2018). Notably, the data on incidence and mortality are 100 % similar. All cases of rabies in the 28 African countries within 2005—2018 resulted in death.

DISCUSSION

There is a dearth of information on the rabies incidence and even mortality rates in a lot of African countries. Rabies causes an estimated 21,000—25,000 deaths in Africa [4, 6]. However, due to an absence of awareness in a lot of communities and a lack of instituted control strategies, surveillance data are not reliable and this is a key factor in the negligence in countries with high rabies burden [21]. The WHO, OIE, FAO and GARC have proposed a strategic global plan to end canine mediated human rabies by 2030 [8, 23].

Africa is yet to acknowledge rabies as an urgent public health problem; this may be due to a lack of understanding of the disease burden and poor surveillance. To tackle this, policies should be instituted to raise awareness of rabies right down to the grassroots level and collaboration between necessary agencies to initiate changes in rabies policies [3]. In a global survey carried out in rabies endemic countries, 49 of the 54 African countries were classified as moderate to a high risk for human rabies while, of the 23 countries that responded to the survey, 16 had ineffective surveillance systems [26]. The WHO meetings in Geneva held in 2018 on "Driving progress towards rabies elimination" pointed out that political commitment is a crucial factor as governments would drive the coordination of the elimination strategies and provide leadership.

The highest combined rabies incidence and mortality in countries for the time range was 1601 in 2006 while the lowest was 157 in 2005. Mozambique had the most cases of rabies (92.9 %) in 2006 which was also the year with the highest rabies cases and mortality. This was worrisome as it denoted a huge absence of surveillance and prevention policies. The rabies incidence in Mozambique increased from 43 in 2005 to 1487 in 2006 and declined to 53 in 2007.

Kenya had the highest number (2262) of rabies cases and mortality throughout the time period (2005—2018). Kenya's rabies incidence and mortality has been greater than 700 every year since 2016; this is despite of the fact that Kenya instituted a rabies elimination strategy in 2014. This may be due to factors such as low vaccination as vaccination coverage in Kenya was only 0.5 % [6]. The increased use of dogs as guard and companion animals may have led to an increased number of dog bites and thus the transmission of rabies, especially in areas where dogs roam freely [9, 27]. The cost of treating for dog bites has been raised because of post exposure prophylaxis and rabies immunoglobulin utilization in human health facilities [15].

Five countries—Angola, Central African Republic, Kenya, Mozambique and Senegal had rabies cases and mortality at 65 % of all rabies cases. Guinea-Bissau, Tunisia and Zimbabwe had a fairly low constant rabies incidence and mortality with the highest number of cases less than 20 per year. These figures may be due to low reporting and surveillance and thus lower data on rabies cases.

The findings from the data obtained showed that all reported cases of rabies led to death in the investigated period. This may be because bite victims may delay in seeking medical treatment either due to ignorance, cost of treatment or unavailability of treatment options. Also, this shows there is a lot to be done in the rabies prevention and treatment in the research area.

The epidemiology of rabies in Africa is not well defined; thus, government support for its control is low. Authorities may consider rabies control to be bogus as the task and cost involved for vaccinating numerous owned and stray dogs is high and so is the level of vaccination coverage needed for rabies control [11]. Furthermore, Africans needs to lead this change as they are one of the worst hit by the rabies virus [16]. Thus, rabies control is not a priority when compared to other diseases of public health concerns. Effective surveillance systems are necessary and that is the major challenge especially in rural areas. Effective surveillance systems include some key features; first rabies has to be regarded as a notifiable disease; this would aid collation of information and allow authorities better monitoring of this disease. Health care professionals should be trained in surveillance methods and diagnosis of the disease according to internationally recognized standards. The active involvement and participation of the local community is also a key feature. They must be made aware of the disease and its risks to encourage reporting of suspected cases and early treatment. Furthermore, following the significant findings of recent studies (17—20), more work needs to be carried out to identify effective vaccines for rabies.

Limitations

Data was not available for a number of years, hence more sophisticated analysis could not be carried out.

Under-reporting of diseases is prevalent in Africa, hence this might not completely reflect the true state of rabies incidence and mortality.

Recommendations

More work should be devoted to research on rabies prevention and cure.

Policies should be put in place to enable good and consistent data. This would contribute toward positive decisions on issues related to rabies.

CONCLUSIONS

Rabies is a fatal zoonotic disease and greatly affects African and Asian countries. The data on rabies which describes practices and policies are very sparse. The analysis of the existing data on rabies incidence and mortality from 28 African countries was conducted which demonstrated that all cases of rabies led to death within the study period (2005—2018). There is therefore a lot to be done to put this fatal and deadly disease at bay.

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THE EFFECT OF CO-ADMINISTRATION OF INACTIVATED VACCINES AGAINST BOVINE RESPIRATORY DISEASE AND NEONATAL CALF DIARRHOEA

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ABSTRACT

A pilot study was performed to evaluate the safety and serological responses after co-administration of two multivalent inactivated vaccines to pregnant cattle. One vaccine was directed against bovine respiratory disease (BRD) and contained antigens of bovine respiratory syncytial virus (BRSV), parainfluenza 3 virus (PI3) and *Mannheimia haemolytica* (Mh). The second vaccine targeted neonatal calf diarrhoea (NCD) and was composed of inactivated antigens of bovine rotavirus (BRV), bovine coronavirus (BCV) and *E. coli*. The use of these combinations have been used more and more by veterinary practitioners as there exist some clear evidence that both vaccines improves the passive protection via the colostrum for the relevant pathogens. However, up until now, no safety or efficacy data has been available concerning such co-administrations. The safety of both vaccines and the serological responses to the BRD vaccine has been evaluated when used at the same time, but without mixing and compared to the responses to the administration of each vaccine independently. There was no evidence of any negative effect on calving or calf health in any of the vaccinated animals. The antibody levels against BRSV and Mh in the sera of the calves from cows vaccinated with both vaccines were not significantly different from the levels in the sera of calves vaccinated with the BRD vaccine alone. The results from this pilot study demonstrated that the co-administration of the two multivalent inactivated vaccines had no detrimental effect on the safety or serological responses to the BRD vaccine compared to the independent use of the vaccines.

Key words: bovine respiratory disease complex; cow vaccination; neonatal calf diarrhoea; passive immunity

INTRODUCTION

Neonatal calf diarrhoea (NCD) and bovine respiratory disease (BRD) are the most important health problems in calves during the first month of life [2, 13]. Both diseases are multifactorial and co-infections with two or more pathogens are common. The list of pathogens associ-

ated with NCD includes bovine rotavirus (BRV), bovine coronavirus (BCV), *E. coli* and *Cryptosporidium parvum* [1, 5, 17]. In the case of BRD, the prevalence vary between studies, but bovine respiratory syncytial virus (BRSV) and *Mannheimia haemolytica* (Mh) seem to be most often involved [8].

In addition to the cost of mortality and treatment of the sick calves, the economic consequences of calf-hood diseases may include reduced growth performance, as well as increased age and difficulty at first calving and reduced milk yields [14, 15, 16]. Given the increased public pressure to reduce intensive antimicrobial use in farm animals, vaccination is an efficient measure to reduce the use of antibiotics in the treatment and prevention of BRD and NCD.

The importance of colostrum has not only been demonstrated for the protection against NCD, but also against BRD [19]. Cow vaccination is widely applied for the prevention of NCD and more recently, the beneficial effects against BRD has been reported [9, 11, 12].

In order to obtain the highest antibody levels in the colostrum, the cows must be vaccinated shortly before calving. The possibility for the co-administration of a BRD and a NCD vaccine would simplify the herd vaccination schedules and reduce the handling of the animals.

In general, no information is available on the safety and efficacy of the co-administration of separately licensed vaccines. Therefore, regulators advise that a decision needs to be made on a case by case basis. In the pilot study reported here, the safety and serologic response to BRSV and Mh after co-administration of two multivalent inactivated vaccines to pregnant cattle was investigated. One vaccine was directed against BRD and contained antigens of BRSV, parainfluenza 3 virus (PI3) and Mh. The second vaccine targeted NCD and was composed of inactivated antigens of BRV, BCV and *E. coli*. The antibody levels against PI3 were not measured, as any negative effects from the co-administration on the serological response to the BRD vaccine is likely to be more pronounced for the other two antigens measured [10].

MATERIALS AND METHODS

The United Kingdom Home Office controls experiments and scientific procedures which may have the effect of causing the animals pain, suffering, distress or lasting harm and does so by the issue of licenses under the Animals (Scientific Procedures) Act 1986. Moredun Scientific Limited conducts its work under Home Office License Numbers PPL/60/3884 and PPL/60/3747. These licenses strictly specify the limits of severity of effects on the animals.

Study design

The purpose of this study was to evaluate the safety and BRD specific serological responses of two commercial inactivated multivalent vaccines for cattle when administered concurrently to pregnant cattle. In addition, an assessment of the safety of the co-administration to pregnant animals was carried out, with additional observations on the vaccinated animals' offspring.

Animal Husbandry

The animals (Holstein Friesian) were housed at a conventional dairy farm in accordance with the standard husbandry practices. The study animals were co-mingled and housed in the same accommodation as non-study animals. The cows were fed a complete mixed diet for pregnant dairy cows (mixture of grass silage, whole crop wheat, brewers' grain, concentrate pellets and molasses). Animals in lactation received a ration of concentrates at each milking. Free access to hay/silage and clean water was available at all times.

Prior to Day 0, thirty pregnant cows were allocated to the treatment groups (10 per treatment group), ensuring that each treatment group contained an even spread of animals with a range of BRSV antibody titres (based on pre-study screening). Calves were left with their mothers for the first 24 hours following birth and then fed with commercial milk replacer.

Vaccinations

All vaccinations were given approximately 3 to 6 weeks prior to the expected calving dates (D0). Animals in test group 1 (T01) were administered 2 ml of a vaccine against neonatal calf diarrhoea (NCD). The vaccine contained inactivated antigens of bovine rotavirus (BRV), bovine coronavirus (BCV) and *E. coli* (Rotavec® Corona; MSD-Animal Health, Boxmeer, The Netherlands) and was applied by intramuscular injection into the proximal area of the neck. Animals in test group 2 (T02) were vaccinated subcutaneously with 5 ml of an inactivated bovine respi-

ratory disease (BRD) vaccine containing a BRSV-PI3-Mh combination (Bovilis® Bovipast RSP; MSD-Animal Health, Boxmeer, The Netherlands). The injections were given into the distal area of the neck. Animals from test group 3 (T03) were concomitantly administered 2 ml of the NCD vaccine by intra-muscular injection into the proximal area of the neck, and 5 ml of the BRD vaccine by subcutaneous injection into the distal area of the neck at a site distinct from the site where the other vaccine was applied. All injections were administered to the right side of the neck. Commercial vaccine batches released according to the standard requirements of the manufacturer were used.

Evaluation of safety parameters

Clinical observations and injection site assessments were carried out following the administration of the vaccines. The observations consisted of a measurement of the rectal temperature from Day 2 to Day 4 and an assessment of the general condition of the animals from Day 2 to Day 4 by experienced personnel once daily, and by a veterinarian prior to vaccination on Day 0. The injection site assessments were carried out by experienced personnel once daily for all study animals from Days 0 to Day 7, and then twice weekly until the reactions had resolved (up to Day 28) or upon calving. The injection site assessments consisted of the following observations at the injection sites and surrounding area: presence of swelling, size of swelling measured by a calibrated ruler (mm: length × breadth), and the type of swelling (e. g. oedema, firmness). The calves were observed within 24 hours of birth, to determine whether they were free from abnormalities and were in good general health.

Measurement of BRSV and Mh antibody responses

Non-heparinized blood samples were collected from each cow: prior to administration, within 24 hours after calving, and from each calf within 24 hours after birth. The antibody levels against BRSV and Mh were determined by quantitative ELISA. The BRSV assay was an in-house ELISA. The *M. haemolytica* A1 antibody ELISA was performed as described previously [10].

First, all plates were coated. The positive and negative detergents extracted coating antigens were diluted in ELISA coating buffer (pH 9.6) and mixed well. The working dilution of the coating antigens is detailed in Table 1 and was pre-determined by a checkerboard titration.

Table 1. Working dilution of the coating antigens in antibody BRSV ELISA test

	Reagent	Dilution
Coating Antigen	Positive: BRSV Rispoval	1/800
	Negative: BRSV BEK Control	1/800
Positive Control	M989	High 1/100 Low 1/1100
Negative Control	FBS	1/50
Conjugate	Rabbit α Bovine HRP	1/5000
Substrate	Enhanced K-Blue TMB	Neat
Stop Solution	0.25 M Sulphuric Acid (H2SO4)	Neat
Coating Buffer	Sodium Carbonate/Bicarbonate Buffer pH 9.6	N/A
Wash Fluid	1xPBS/0.05 % Tween 20 (PBST)	N/A
Diluent	1xPBS/Tween 20/EDTA/0.55 % ovalbumin (PBSTOval)	N/A

100 µl of the positive antigen was added to all of the wells in rows A, B, E and F of a medium binding ELISA plate (Greiner M129A) and 100 µl of negative antigen to all of the wells in rows C, D, G and H. The coated plate was sealed and incubated overnight at +2 °C to +8 °C. The ELISA diluent (PBST/Ovalbumin) was prepared by adding 2 ml of 0.1 M EDTA to 178 ml of ELISA wash fluid (PBST). Approximately 20 ml of this solution was added to 1 g of ovalbumin powder in a universal and stirred to dissolve the ovalbumin. A Buchner funnel was prepared using Whatman No. 3 filter paper—filter was pre-wetted with some of the remaining PBST/EDTA solution and the 20 ml ovalbumin solution was filtered through the filter and the filtrate was added to the remaining 160 ml of PBST/EDTA solution. The ELISA coating antigen was discarded from the plate. The plate was washed 4 times with ELISA wash fluid (PBST) and the plate was blotted dry. The test serum was diluted 1 : 50 in the appropriate sample dilution tube by adding 10 µl of sample to the pre-aliquoted 490 µl of ELISA diluent (PBST/Ovalbumin). Positive and negative control sera were diluted in ELISA diluent (PBST/Ovalbumin). 100 µl of positive and negative control sera dilutions and 100 µl of diluted test sample were added to the appropriate 4 wells (2 positive and 2 negative) of the coated ELISA plate. The plate was sealed and incubated for 1 hour (+10 minutes) at 37 °C (+2 °C). The plate was washed 4 times with ELISA

Table 2. Mean rectal temperature and percentage of cows with a rectal temperature exceeding 39.0 °C before and after vaccination

Group	Days before/after vaccination						
	-2	-1	0	1	2	3	4
	Mean Rectal Temperatures [°C]/Percentage cows > 39.0 °C						
NCD	38.5/0	38.5/0	38.6/0	38.7/0	38.6/20	38.4/0	38.4/0
BRD	38.4/0	38.6/0	38.7/10	38.8/30	38.5/10	38.5 0	38.5/10
Co-administration	38.5/10	38.5/10	38.6/10	39.0/50	38.6/10	38.4/10	38.4 0

Mean rectal temperatures [°C] and percentage of calves with a rectal temperature exceeding 39.0 °C before and after vaccination with an inactivated NCD vaccine (T01), an inactivated BRD vaccine (T02) or co-administration of both vaccines (T03)

wash fluid (PBST) and blotted dry. 100 µl of diluted Rabbit anti-Bovine Horseradish Peroxidise (RαBovHRP) conjugate in ELISA diluent (PBST/Ovalbumin) was added to all of the wells. The plate was sealed and incubated for 50—70 min at 35—39 °C (+2 °C). The plate was washed 4 times with ELISA wash fluid (PBST) and blotted dry, 100 µl of enhanced TMB substrate was added to all of the wells and incubated at room temperature for 5 minutes to allow the colour to develop. The reaction was stopped by adding 50 µl of Stop solution (0.25 M sulphuric acid) to all of the wells. The plate was read at 450 nm.

The results of the samples were produced as a Dynex Revelation 4.04 printout. This consisted of a Data Matrix/Table; containing the corrected optical density values (ODs) and a Ratio Results table; containing the Ratio Matrix calculated results (Ratio Matrix). The positive and negative results were taken from the Ratio Matrix (RM) values as follows: RM value < 0.100 was negative, RM value ≥ 0.100 and < 0.150 was equivocal (repeat/investigation of another sample approx. 14 days apart), and RM value ≥ 0.150 was positive.

The BRSV and *M. haemolytica* antibody levels for the group vaccinated with the BRD vaccine (T02) were compared with the group vaccinated with both vaccines (T03) and with the group vaccinated with the NCD vaccine (T01). The formula for Student's T-test for two-tailed distribution and two-samples with unequal variance within Microsoft Excel were applied. In the results, the values are given as the mean ± the standard deviation.

RESULTS

Clinical reactions and body temperatures after vaccination

No abnormal observations relating to the application of the vaccines were observed over the four day period post-vaccination (Study Day 0 to Day 4). The body temperatures of some animals were slightly increased after vaccination but remained within the physiological range. Five of the 10 vaccinated animals in T03 had a temperature exceeding 39.0 °C 1 day after vaccination, but the temperature normalized (< 39.0 °C) within 24 hours. See Table 2 for the mean group rectal temperatures and the percentage of cows with a rectal temperature exceeding 39.0 °C before and after vaccination.

Injection site reactions

One animal in the group vaccinated with the NCD vaccine alone and one animal in the group vaccinated with both vaccines had measurable injection site reactions to the NCD vaccine (see Table 3 for the results). The reaction persisted until 28 days post vaccination in the case of vaccination with only one vaccine and disappeared within 24 hours post vaccination in the animal that was given both vaccines.

After the subcutaneous vaccination with the BRD vaccine alone, all 10 animals had a measurable injection site reaction for up to 21 days after vaccination as compared to 8 animals having measurable site reactions for up to 17 days after vaccination in the group that received both vaccines.

Calving results

A total of 27 cows calved successfully after an average duration of pregnancy of 285, 282 and 283 days for the groups vaccinated with the NCD vaccine, the BRD vaccine, or both vaccines, respectively. The three remaining cows gave birth to stillborn calves; one animal vaccinated with the BRD vaccine gave birth to a stillborn calf approximately three weeks before the expected calving date. The findings at the *post mortem* indicated that the abortion was most likely due to an infection with the *Bacillus licheniformis*. A second animal in that group gave birth to a still born calf. Findings at that *post mortem* examination indicated that the calf was born to term and the death was most likely caused by asphyxiation. Twin calves were stillborn slightly before term from one cow of the group vaccinated with both vaccines. No abnormalities were observed at that *post mortem* examination.

Health conditions of calves born alive

All calves born alive were found healthy and remained healthy during the four days observation period with the exception of one calf born from a dam vaccinated with both vaccines (individual data not shown). That calf had an increased respiratory effort from birth onwards. The demeanour was normal. However, on thoracic auscultation a tachycardia was apparent. The calf was euthanized, and a necropsy was performed. Gross findings showed evidence of pneumonia in all lobes of the lung with an abscess in the left antero-dorsal region. It was concluded that the animal was suffering from a pulmonary infection.

Serological responses against BRSV and Mh

All cows were sero-positive for BRSV at the time of vaccination. The BRSV-specific IgG levels were significantly lower at the time of vaccination in cows vaccinated with BRD vaccine alone (T02 1.027 ± 0.182) compared to cows vaccinated with NCD vaccine alone (T01 1.210 ± 0.188) ($p = 0.04$). There was no statistical difference between the mean BRSV-specific IgG levels of other treatment groups at the time of the vaccination (Figure 1A for comparison of mean values) ($p = 0.89$ and 0.12 for respectively T01 vs T03 and T02 vs T03).

The BRSV-specific IgG levels were significantly higher in post-calving cows vaccinated with BRD vaccine alone ($p = 0.003$; T02 1.474 ± 0.396) or co-administration with the NCD vaccine ($p = 0.05$; T03 1.322 ± 0.414) compared to cows vaccinated with NCD vaccine alone (T01 0.950 ± 0.285). Moreover, there was no statistical difference between the mean BRSV-specific IgG levels post calving of the cows vaccinated with the BRD vaccine alone compared to the co-administration ($p = 0.44$) (see Figure 1A for comparison of average values).

All calves were positive for the detection of antibodies against BRSV with the exception of one animal in the group vaccinated with the NCD vaccine alone. However, the BRSV-specific IgG levels were significantly higher in calves born from cows vaccinated with the BRD vaccine alone ($p = 0.02$; T02 1.561 ± 0.383) or co-administered ($p = 0.01$; T03 1.388 ± 0.375) compared to calves born from cows that received only the NCD vaccine (T01 0.904 ± 0.416). Moreover, there was no significant difference in the BRSV-spe-

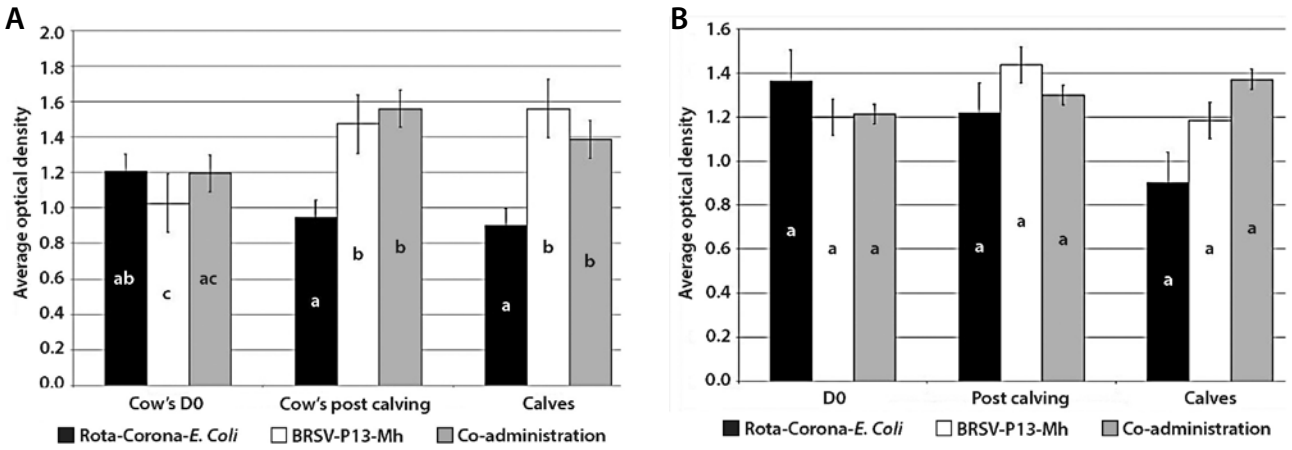

Fig. 1. Antibody response against BRSV and Mh

Antibody response against BRSV (A) and Mh (B) expressed as OD values ± standard error in the sera of the vaccinated animals at the time of vaccination, post calving and in the sera taken from their calves. The cows were vaccinated with an inactivated NCD vaccine (T01) (black bars), an inactivated BRD vaccine (T02) (white bars) or co-administration of both vaccines (T03) (grey bars)

cific IgG levels of calves born from cows that received the BRD vaccine alone or co-administered ($p = 0.32$).

The results of the Mh antibodies were similar to those for the BRSV antibodies; they indicated a trend towards higher titres for the calves from cows vaccinated with both vaccines (T03 1.214 ± 0.372, 1.299 ± 0.346, 1.371 ± 0.481 at respectively day of vaccination, post calving and in calves); however the results were not significantly different from the other groups (see Figure 1B for comparison of average values). All cows were sero-positive for Mh at the time of vaccination and after calving. All calves were positive for the detection of antibodies against Mh with the exception of the calf born to a cow that was vaccinated only with the NCD vaccine that was also negative for BRSV specific antibodies. There was no significant difference in Mh-specific IgG levels after the calving of cows that received the BRD vaccine alone (T02 1.438 ± 0.268) or co-administered ($p = 0.33$; T03 1.299 ± 0.346). Also for the other groups, there was no significant difference in the Mh-specific IgG levels in the cows after calving ($p = 0.18$ and 0.65 for respectively T01 (1.220 ± 0.410) vs T02 (1.438 ± 0.268) and T01 (1.220 ± 0.410) vs T03 (1.299 ± 0.346)). There was no significant difference in the Mh-specific IgG levels of calves born from cows that received the BRD vaccine alone (T02 1.185 ± 0.551) or co-administered (T03 1.371 ± 0.481) ($p = 0.46$). Also for the other groups, there was no significant difference in the Mh-specific IgG levels in the calves ($p = 0.32$ and 0.07 for respectively T01 (0.905 ± 0.606) vs T02 (1.185 ± 0.551) and T01 (0.905 ± 0.606) vs T03 (1.371 ± 0.481)).

DISCUSSION

The pilot study presented here was performed to investigate the safety and efficacy of concomitant application of two multivalent inactivated vaccines in pregnant cattle. The results indicated that the two multivalent inactivated vaccines were tolerated well after co-administration of both vaccines. A transient rise in temperatures is a common observation after application of inactivated bacterial multi-strain vaccines [3, 6]. Inactivated viral vaccines often do not induce hyperthermia [7].

In general, swellings may represent a local reaction against vaccine antigens and/or the adjuvant components contained in the vaccines. Reactions, as seen in this study, have been reported for different kinds of adjuvants [7, 20] and are generally considered as acceptable for cattle vaccines. Most importantly in the context of this study, the number and the duration of local reactions did not increase after concurrent use of both vaccines.

As the pilot study was performed in pregnant animals, the outcome of the pregnancy was the most important safety parameter. For all three cows that gave birth to dead calves, the cause was unlikely to be related to the vaccination. Similarly, it was concluded that the calf with health problems was suffering from either congenital pneumonia or pulmonary infection, both of which are not considered to be related to the vaccinations. Considering all of the safety results together, there were no detrimental effects after the single or concurrent use of the two inactivated multivalent vaccines in pregnant cattle.

The second objective of the study was to evaluate the efficacy of the vaccines when used singly or concurrently. The purpose of cow vaccination is to increase antibody levels in the cow which are then transferred to the calves via the colostrum [4, 18]. Therefore, in the context of this study, the measurement of antibody levels in the calves' sera was an appropriate parameter to judge the efficacy of these vaccines as colostrum management was appropriate.

The results of BRSV and Mh antibodies in the calf sera clearly indicated that the vaccinations induced a specific immune response in the cows and, most importantly, the antibody response in the calf sera were not significantly different between the groups vaccinated with the vaccine against bovine respiratory disease alone or with both vaccines.

CONCLUSIONS

The results of this pilot study are promising, but further studies are required to fully demonstrate the safety and the efficacy after co-administration of these two vaccines and the value of passive immunity against respiratory pathogens.

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CONFLICT OF INTEREST

The authors Birgit Makoschey and Geert Vertenten declare conflict of interest. They are employees of MSD Animal Health, the company that markets the vaccines that have been used in the study reported herein.

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IN VIVO ASSESSMENT OF ZEARALENONE TOXICITY

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ABSTRACT

The microscopic filamentous fungi of the genus *Fusarium* are capable of producing secondary metabolites—mycotoxins. *Fusarium fungi* synthesize trichothecenes, zearalenone (ZEA) and fumonisins under appropriate environmental conditions. In this biological experiment, we studied the effects of zearalenone on a model organism called *Artemia franciscana*. During the three-day *in vivo* tests, we used five different concentrations of zearalenone (0.08 ppm, 0.4 ppm, 2 ppm, 10 ppm and 50 ppm). The results of this study showed that as the zearalenone concentration and the duration of the mycotoxin exposure increased, the lethality of artemia also increased. Our study showed that the toxicity of zearalenone to *Artemia franciscana* was relatively low.

Key words: *Artemia franciscana*; *in vivo*; toxicity; zearalenone

INTRODUCTION

Zearalenone (ZEA) is one of the most important mycotoxins and it is produced by the *Fusarium* toxigenic species: *F. graminearum, F. culmorum, F. equiseti, F. crookwellense, F. roseum, F. nivale, F. tricinctum, F. sporotrichioides, F. oxysporum, F moniliforme, F. lateritium, F. sacchari, F. sambucinum, F. gibbosum* and others [2, 14]. Zearalenone (Fig. 1) is a non-steroidal estrogenic mycotoxin that has a structure similar to steroid hormones, increasing its ability to bind to the intracellular oestrogen receptors of the uterus, hypothalamus and pituitary gland. It acts as an agonist and partly as an estradiol antagonist. As a result of this action, zearalenone inhibits the secretion of the follicle-stimulating hormone (FSH) and suppresses the maturation of ovarian follicles in the preovulatory phase [20]. In terms of toxicity, the ZEA is responsible for the functional changes in the reproductive system with subsequent overall disruption of hormonal regulation. In addition to its estrogenic effects, it may also be hepatotoxic, haematotoxic, immunotoxic and genotoxic [34].

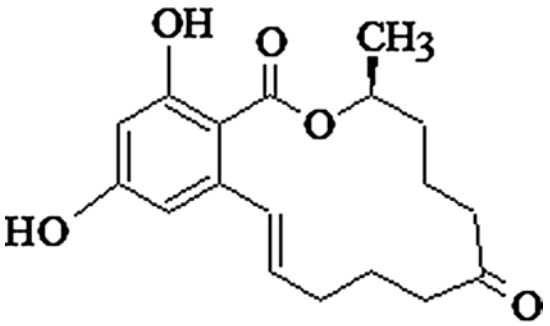

Fig. 1. Chemical structure of zearalenone

Chemically, zearalenone is a macrocyclic lactone of β-resorcylic acid and has a close structural relationship with other antibiotic metabolites that are produced by a number of microscopic filamentous fungi [6]. Zearalenone is a white crystalline substance with a melting point of 159—163 °C. Its chemical name is 6-(10-hydroxy-6-oxo-trans-1-undecenyl) β-resorcylic acid lactone. The molecular formula of zearalenone is C18H22O5 and the molecular weight is 318.36 g.mol^{-1}. ZEA is a relatively stable substance that is not subject to degradation in the cereal processing or heat treatments. It is soluble in benzene, acetonitrile, methanol, ethanol and acetone [8].

Effects of ZEA on the domestic animals

Pigs are particularly sensitive to ZEA compared to other animal species, because their liver undergoes a biotransformation of zearalenone and consequently the synthesis of α-zearalenol, which is more toxic than zearalenone alone. Compared to zearalenone and β-zearalenol, α-zearalenol has a higher ability to bind to oestrogen receptors [19]. As early as in 1927, a disease called Zearalenone-syndrome was observed in pigs after the feeding of mouldy corn. The main symptoms of the Zearalenone-syndrome in pigs included changes in the genital system such as oedema and vulvar inflammation, vaginal and rectal prolapse and enlargement of the mammary gland [9]. G i m e n o and Q i u n t a n i l l a [10] reported a case of zearalenone intoxication flare up on a horse farm. Among the most observed symptoms in mares were feed rejection, uterine prolapse and internal haemorrhages. In males, in turn, a sharp weakening of the genital apparatus was observed. Various sources have reported that the cases associated with zearalenone intoxication in ruminants were mainly related to its estrogenic effect and included, in particular, abortions in females [16, 17, 22]. Other symptoms include: vaginitis, increased vaginal secretion, decreased reproductive capacity, and enlarged mammary glands in heifers [32]. In poultry, in addition to neck oedema, ovarian cysts have also been observed [1]. In young turkeys, comb oedema, inflammation of the anus and enlargement of the ovaries have been described. The influence of libido and adverse effects on spermatogenesis has been observed in ganders and cocks, and fertility has been reduced in laying hens [9]. In dogs and cats, a local contact mucosal irritation has occurred following zearalenone intoxication. Upon transfer of zearalenone to the body, mycotoxin acts as a false estrogen, causing acute vulvovaginitis, disturbances of the estrous cycle and impaired fertility [30]. The estrogenic effects of ZEA caused several reproductive disorders in animals, as well as hyperestrogenic syndromes in humans [24].

Occurrence of zearalenone in feed and food

Zearalenone is worldwide [2] and contaminates about 32 % of cereals and their products (biscuits, breakfast cereals, pastries, desserts) [4]. It is found mainly in corn, oat, rye, rice, sorghum and wheat [5, 33].

Zearalenone classification according to IARC

According to the International Agency for Research on Cancer (IARC), zearalenone belongs to group No. 3, which includes mycotoxins that are not classified as carcinogenic to humans [15]. However, zearalenone is currently covered by the Commission Regulation (EC) No. 1881/2006, which sets the maximum levels for certain food contaminants.

MATERIALS AND METHODS

We used commercial zearalenone (Sigma Aldrich, Schnelldorf, Germany) for experimental purposes. The biological activity of zearalenone was monitored *in vivo* on a model invertebrate organism—*Artemia franciscana*. The conditions for the optimal hatching of artemias were prepared according to D v o ř á k et al. [7]. The natural environment of *Artemia franciscana* is the inland salt water; so it was necessary to prepare "seawater", the chemical composition of which is given in Table 1.

Dried cysts "Maxima brine shrimp eggs" (Sanders, Utah, USA) were added to the prepared seawater. Favour-

Table 1. Chemical composition of the seawater

Chemicals	Weight [g.l^{-1}]
NaCl	23.900
$MgCl_2.6H_2O$	10.830
$CaCl_2.6H_2O$	2.250
KCl	0.680
$Na_2SO_4.10H_2O$	9.060
$NaHCO_3$	0.200
$SrCl_2.6H_2O$	0.040
KBr	0.099
H_3BO_3	0.027

able hatching conditions were ensured by the sufficient oxygen supply and constant egg movement, using a Maxina vibrating membrane compressor (Hagen, USA). The optimal hatching rate of artemia was achieved according to Sanders instructions in 18 hours at a temperature of 27—30 °C, a salinity of 1.2—3.0 % and a pH of 7.5—8.5. Since hatching was performed at 25 °C, it was extended to 24 hours.

Zearalenone toxicity test procedure

In the three-day toxicity tests, we used the methodology of D v o ř á k et al. [21]. For each test, we used 10 nauplii stages of *Artemia franciscana*, which were transferred to a Petri dish (60 mm diameter) containing 10 ml of seawater using a Pasteur pipette. The toxic effects of 5 concentrations of zearalenone (50 ppm; 10 ppm; 2 ppm; 0.4 ppm; 0.08 ppm) were observed. Since zearalenone is not water soluble, DMSO was used for dilution; the final concentration in seawater being 1 %. Each zearalenone concentration was tested on a set of 50 individuals, divided into five Petri dishes (à 10 nauplii). The viability of artemia was compared to the two control groups. The first control group (50 artemias divided into 5 Petri dishes of 10 individuals in 10 ml of seawater) served as a workflow control. A second control group (50 individuals divided into 10 pieces of 5 petri dishes) was placed in 10 ml of sea water solution containing 1 % DMSO (DMSO effect control). A constant temperature of 22 °C was ensured by placing the samples in a thermostat during the experiments. The survival of artemia was observed after 24, 48 and 72 hours. We considered living artemia to be individuals that demonstrated movement compared with those that did not move any part of their body. The viability of artemias was observed using a magnifying glass against a black background.

Statistical evaluation

The data obtained in this study were reported as means ± standard deviation (SD) and analysed using ANOVA and the Dunnett's test. The statistical significance among parameters was considered at $P < 0.05$.

RESULTS

By using the *in vivo* toxicity test of zearalenone, we observed its toxic effect on the model organism of the *Artemia franciscana*. The lethality of artemia induced by different concentrations of zearalenone, as a function of exposure time is presented in Table 2. The results of the statistical analysis showed that as the zearalenone concentration and the duration of exposure increased, the lethality of artemia also increased.

Compared to the control group (0 ppm), a significant increase in the lethality of artemia was observed after a 24-hours exposure to zearalenone at 10 ppm (16 %, $P < 0.001$) and 50 ppm (12 %, $P < 0.001$). After a 48 h exposure, a statistically significant death of artemia was observed at concentrations of: 0.4 ppm (8 %, $P < 0.01$), 2 ppm (10 %, $P < 0.001$), 10 ppm (16 %, $P < 0.001$) and 50 ppm (18 %, $P < 0.001$). Also, after 72 hours, the lethality of artemias was significantly increased at zearalenone concentrations of: 0.4 ppm (8 %, $P < 0.05$), 2 ppm (12 %, $P < 0.01$), 10 ppm (18 %, $P < 0.001$) and 50 ppm (24 %, $P < 0.001$).

The determination of LD50 (Probit Analysis) at the tested zearalenone concentrations was not possible due to the low lethality of *Artemia*.

DISCUSSION

At present, an *in vivo* test on the aquatic invertebrates of the genus *Artemia* has been used to verify the toxic effects of various substances. This assay was designed by M i c h a e l et al. in 1956 [21]. The model organism for re-

Table 2. The lethality [%] of *Artemia franciscana*

ZEA [ppm]	24 h		48 h		72 h	
	x ± SD	L [%]	x ± SD	L (%)	x ± SD	L [%]
0	10.0 ± 0	0	10.0 ± 0	0	10.0 ± 0	0
0.08	10.0 ± 0	0	10.0 ± 0	0	9.6 ± 0.55	4.0
0.4	9.4 ± 0.55	6.0	9.2 ± 0.45 **	8.0	9.2 ± 0.45 *	8.0
2	9.4 ± 0.55	6.0	9 ± 0 ***	10.0	8.8 ± 0.45 **	12.0
10	8.4 ± 0.55 ***	16.0	8.4 ± 0.55 ***	16.0	8.2 ± 0.45 ***	18.0
50	8.8 ± 0.84 **	12.0	8.2 ± 0.45 ***	18.0	76 ± 0.55 ***	24.0

ZEA—zearalenone; x—mean number of live individuals; SD—standard deviation; L—lethality of artemia; significance of differences compared to control sample: *—P < 0.05; **—P < 0.01; ***—P < 0.001

search fields such as environmental ecotoxicology, genetics, and evolutionary studies continues to be *Artemia* spp. [3, 23]. The principle of these tests is based upon the lethality of *Artemia* after the treatment with the test substance as a function of concentration and exposure time [25]. It is used to test the biological activity of plant extracts [31], cyanobacterial toxins [13] and has previously served as a screening test for heavy metal toxicity [29], pesticides [21] and fungal mycotoxins [12].

To assess the elemental toxicity of hazardous substances under *in vivo* conditions, a bioassay using artemia aquatic animals is currently preferred [11, 18], because it is a simple and inexpensive test using a large number of organisms for statistical validation and no special equipment is needed [25]. Biological assay with artemia does not require animal serum, therefore it prevents unnecessary use of animals in scientific experiments [26].

The best studied of the *Artemia* species is *Artemia franciscana*. It represents the vast majority of studies in which *Artemia* is used as an experimental test organism [28]. Our results of toxicity testing of zearalenone on the model organism *Artemia franciscana* indicated a relatively low level of zearalenone toxicity. The highest lethality (24%) was seen after 72 hours exposure to zearalenone at a concentration of 50 ppm. Similarly, the low lethality of artemia (18%) was also observed by H a r w i n g and S c o t t [12], who used a zearalenone concentration of 10 ppm in their experiments. However, when tested for the toxic effect of fumonisin B2 at 66 ppm, H a r t l and H u m p f [11] found nearly 100% lethality of artemia after 48 hours exposure. According to R i c h a r d s o n and H a m i l t o n [27], the brine shrimp assay is suitable for the prediction of trichothecene toxicity in broiler chicks. The low lethality of *Artemia franciscana* after exposure may be explained by the fact that zearalenone, as a mycotoxin with estrogenic effects, primarily binds to oestrogen receptors and is responsible for functional changes in the reproductive system, with subsequent disruption of hormonal regulation [20, 34].

CONCLUSIONS

The occurrence of secondary metabolites of microscopic filamentous fungi is a global problem. They cause humans and animal mycotoxicosis. Zearalenone causes functional changes in the reproductive system, therefore constant investigations of this toxic substance is essential. This study pointed to the low lethality of the model organism *Artemia franciscana*. Further biological experiments are needed to determine the effects of zearalenone on the reproductive apparatus of *Artemia* spp.

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HISTORY OF DNA SEQUENCING

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ABSTRACT

The nucleotides are the building blocks of nucleic acids and determining their sequential arrangement had always been an integral part of biological research. Since the past seven decades, researchers from multidisciplinary fields has been working together to innovate the best sequencing methods. Various methods had been proposed, from some oligonucleotides to the whole genome sequencing, and the growth had gone through adolescence to the mature phase where it is now capable of sequencing the whole genome at a low cost and within a short time frame. DNA sequencing has become a key technology in every discipline of biology and medicine. This review aims to highlight the evolution of DNA sequencing techniques and the machines used, including their principles and key achievements.

Key words: DNA; NGS; RNA; sequencing; Sequencing machines

INTRODUCTION

We are witnessing the emergence of the cutting edge techniques in biological research that have gained a significant place in reducing the time and cost to obtain biological knowledge. Decades ago, the sequencing technology was time-consuming, labour-intensive, and relied on analytical chemistry. In 1976, the first single-stranded genome of the bacteriophage ØX174 was sequenced using a plus-minus sequencing method. Since then, several genomes (including the human genome) were drafted using the Sanger sequencing method which requires an enormous investment of time and cost [17, 19]. The most popular and highly used technique for DNA sequencing was established by Fred Sanger and it was referred to as the chain termination or dideoxy method. The Sanger method of sequencing has been characterized as the first-generation of sequencing. This phase of development can be considered as an adolescence period, when the human genome project was completed in 2003, whereas from 2007 onwards, the maturing phase was beginning.

There were continuous improvements in the sequencing methods which further led to high throughput sequencing which was collectively called as the next generation of sequencing. During this developmental journey, commercial platforms (such as ABI, Solexa, Ion Torrent, Illumina, Oxford Nanopore, etc.) with different sequencing strategies and concepts were developed, while the common sequencing steps remained conserved such as: the template preparation, clonal amplification and a cyclic round of massively parallel sequencing [17]. These sequencing platforms were capable of producing a huge amount of biological data in less time and money. These developments have opened new perspectives in the area of genomics, transcriptomics and metagenomics.

First-generation sequencing

The conceptual base for the replication and protein encoding by the nucleic acids was supported by the groundbreaking discovery of the three-dimensional structure of DNA by Watson and Crick [25] using photograph 51, produced by the Rosalind Franklin and Maurice Wilkins [26]. Still, the order of four nucleotides was unapproachable as the DNA molecule is longer and composed of only four nucleotide bases that made it difficult to sequence [6]. An initial study in the field of rapid sequencing was carried out in 1970 by Ray Wu. Then in early 1975, the first complete genome was sequenced at the RNA level; this involved the RNA bacteriophage MS2 [5, 19]. Primarily, the focus was on the pure species of RNA such as: transfer RNA, ribosomal RNA and the genome of single-stranded RNA bacteriophages; this was because those are abundant in cell culture, they are shorter, and not complicated with the complementary strands.

In the process of identification of small hypothetical DNA sequence, the first step was polymerization and elongation of DNA sequence using an already known short nucleotide (decamer). The radiolabeled complementary strands of unknown DNA template of various lengths were formed by incorporating four deoxynucleotide triphosphates (dNTPs), in which one was radiolabelled (32P). The second step was the removal of excess triphosphate using an agarose column and this mixture was further used for the minus and plus method [20].

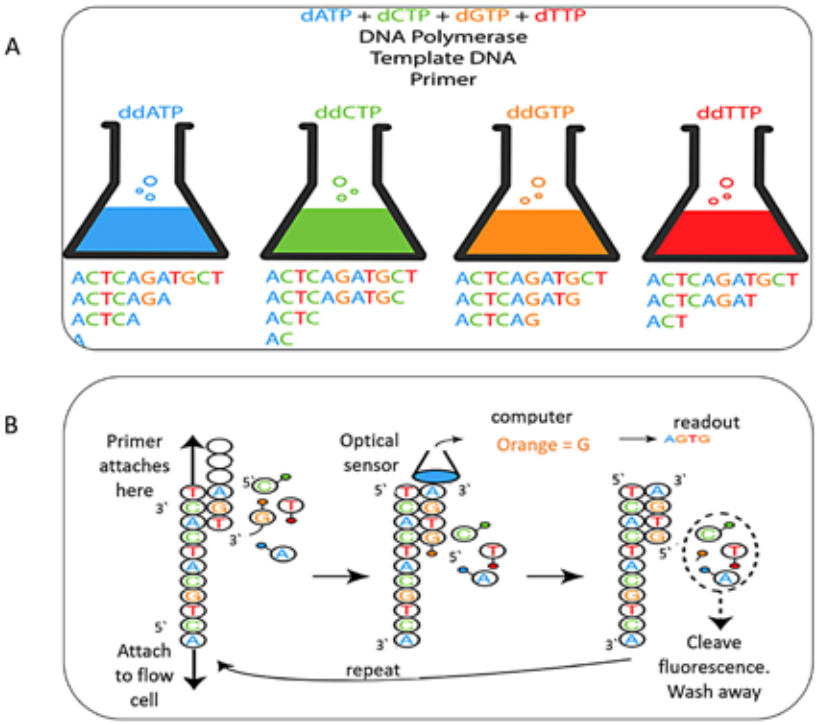

Fig. 1. Pictures briefing the chemistry and techniques used in the evolution of first and second generation DNA sequencing
(A)—The Sanger chain termination methods using types of ddNTPs with DNA polymerase in four separate reactions to infer the DNA sequence (Image Source- Snipcadmy.com); (B)—Illumina sequencing by synthesis techniques, each freely available nucleotide added recognized by the optical sensor and connected to a computer to readout the nucleotide pattern (Image source—Medium.com)

In the minus method, the random mixture of radiolabeled complementary strands was incubated with polymerase I in the presence of three deoxyribose-triphosphates (dNTPs), whenever in the synthesis of DNA, a triphosphate missed, chain terminated at 3' end before that specific residue. The four incubation mixture was synthesized by missing one triphosphate among the four each time and further denatured and subjected to gel electrophoresis for molecular size-based separation. In the case of the plus method, the above obtained random mixture was incubated with only a single type of deoxynucleotide triphosphates and T4 DNA polymerase, because of T4 polymerase exonuclease activity, all extension ended with that triphosphate present. Further, the radioautograph produced from the plus and minus method was used to infer the positions of the nucleotides in the hypothetical DNA sequence [20]. However, this technique was limited to approximately 50—100 nucleotides that consisted of small stretches of DNA and involved lots of analytical chemistry and fractionation steps [7].

After the development of the plus and minus methods, the first rapid sequencing was developed by Gilbert and Sanger with chemical cleavages and chain termination, respectively [11, 19]. In the chain termination method, the DNA monomeric unit (deoxyribonucleic acid mimicked by the chemical analogue di-deoxyribonucleotide (ddNTPs) that lacked a hydroxyl group at 3' prime) which was required for the extension of the DNA chain, resulted in the hindrance of the bond formed between the 5' phosphate of the next dNTP [19]. Whereas, an alternative method (the Maxam and Gilbert chemical cleavage method) involved complex chemistry, in which the double-stranded or single-stranded DNA was first digested with the restriction enzymes and then the end-labelled with 32P phosphate subjected to the random cleavage at adenine (A), cytosine (C), guanine (G) or thymine (T) positions using specific chemical agents [11]. The products of these four reactions were then separated using polyacrylamide gel electrophoresis to inferred the DNA sequence. Due to the benefits of the less toxic chemicals and less complex procedure, the Sanger method was further adopted and modified by the replacement of phospho-radiolabelling with the fluorometric based detection and capillary-based electrophoresis technique to increase the capability.

Gradual refinement contributed to the development of the first-generation automated sequencing machine, however, these machines were only capable of producing reads of less than 1 kilobase length [6]. To address this limitation and to sequence longer fragments of DNA, researchers came up with the shotgun strategy, in which the overlapping regions in the genome were fragmented, cloned using a vector and then sequenced separately and reassembled using computational tools [16]. The overall advances in the sequencing technology led to the enablement to draft the first human genome sequence on 14 April 2003 using the Sanger chain termination method (Fig. 1). The automated DNA sequencer ABI prism 3700 with 96 capillaries was used for this genome sequencing [16, 24].

Second-generation sequencing

The first-generation sequencing (mainly the Sanger sequencing technique) continued to dominate the sequencing market for approximately two decades; however, the researchers were in search of a better alternative technique that would have lower cost, higher throughput and capable of massively parallel sequencing (Table 1). Rather than a chain termination method, a new sequencing method had evolved based on the production of light, whenever a nucleotide was incorporated the release of a pyrophosphate occurred, hence it was called pyrosequencing. During the synthesis of DNA, nucleotides were incorporated by the polymerase enzyme and each incorporation released a pyrophosphate. This pyrophosphate was then, in the presence of ATP sulfurylase and adenylyl sulfate, converted to ATP and then this ATP was used as a substrate for luciferase to produce light that was proportional to the amount of pyrophosphate. In this method at a time, one type of dNTPs was added and if complementary nucleotide found on the unknown DNA template, the light emits. This process further continued with washing and adding different dNTPs to inferred the DNA sequence in real-time [16]. The pyrosequencing technique came into existence in 1993 and was commercialized in 1997 by a company (Pyrosequencing AB) owned by Pål Nyrén and colleagues [15]. This method was not dependable upon electrophoresis and fragment separation, hence it was more rapid than the chain termination. The second-generation sequencing can also be termed as a short-read sequencing approach and can be broadly divided into Sequencing-by-ligation, Sequencing-by-synthesis and Ion semiconductor sequencing [8].

In 1998, Pål Nyrén and colleagues used one more en-

Table 1. Showing the DNA sequencing companies with the details of their platforms and their pros and cons

Company	Sequencing Principle	System platform	Read length and accuracy	Pros	Cons
Illumina	Reversible terminator sequencing by synthesis	HiSeq 2500/1500	36/50/100 SE and > 99 %	Very high throughput, cost-effective, steadily improving read length	Long run time, short read length
		MiSeq	35/50/75/100 SE > 99 %	Short run time, cost-effective, high coverage	Short read length
Roche	Pyrosequencing	454 GS FLX+	1 Million, 99.97 %	Longer reads, high throughput, high coverage	High reagent cost, the higher error rate in homopolymers region
Helicos Biosciences	Single-molecule sequencing	HeliScope	25—55 (average—32) 99.99 %	Non-bias representation of a template for genome	Expensive instrument, very short read length
ABI Life technologies	Ligation	5500 SOLID	75+35 99.99 %	Low reagent cost and high throughput	Long run time and very short reads
	Proton detection	Ion Personal Genome Machine(PGM)	35/200/400 > 99 %	Short run time, low cost/sample	High reagent cost, the high error rate in homopolymers
Pacific Biosciences	The real-time single-molecule DNA sequencing	PacBioRS	Average 3000 84—85 %	Short runtime, very long read length, low reagent cost	No paired reads, the high error rate
Oxford nanopore	Nanopore exonuclease sequencing	gridION	Tens of Kilobytes 96 %	Extremely long reads, no fluorescent labelling and no optics	4 % error rate, difficult to fabricate a device with multiple parallel pores
	Nanopore sequencing	MinION	Up to 1 Megabyte, 99 %	Longest read length, portable, affordable	High cost/Megabytes, No protocol yet

zyme called apyrase to remove the nucleotides that were not incorporated by the DNA polymerase, hence they established the automated setup for Pyrosequencing. Using the principal of pyrosequencing, 454 pyrosequencing method attached the DNA that was to be sequenced to the solid phase fibre-optic slides which consisted of millions of wells and each well was capable for the separate enzymatic reactions; this achievement boosted the rapid growth in parallel sequencing techniques [18, 21]. One of which was Solexa/Illumina sequencing platform that includes: DNA fragmentation, adapter ligation (library preparation), fixation at the flow cell, clustering (PCR- bridge amplification), adding four types (A/T/G/C) of fluorescently labelled reversible terminating nucleotides and the clusters were exited using laser and signals were detected using coupled-charge diode (CCD) [17]. The advantage of this sequencing method was that it was capable to perform paired-end sequencing that increases the accuracy of the information and helps in mapping reads to the reference genome, later this technique was acquired by Illumina.

The growing field of DNA sequencing witnessed another technique called Ion Torrent (Thermo Fisher Scientific) that utilized a semiconductor sequencing technology. In which the hydrogen ions were released whenever the nucleotide was incorporated in a single strand of DNA shifts the pH of the surrounding solution during the polymerization of DNA and these changes were detected by the sensor on the bottom of each well [22]. Each nucleotide was added with the washing cycle and according to the change in voltage, the sequence of the nucleotide was recorded. Another approach that was used commercially in ABI/SOLiD (Supported oligonucleotides ligation and detection) was the sequencing-by-ligation, not sequencing-by-synthesis, that consists of the attaching an adapter to the DNA fragment, one fragment-one bead complex formation and cloned by PCR emulsion, further processed with purification and

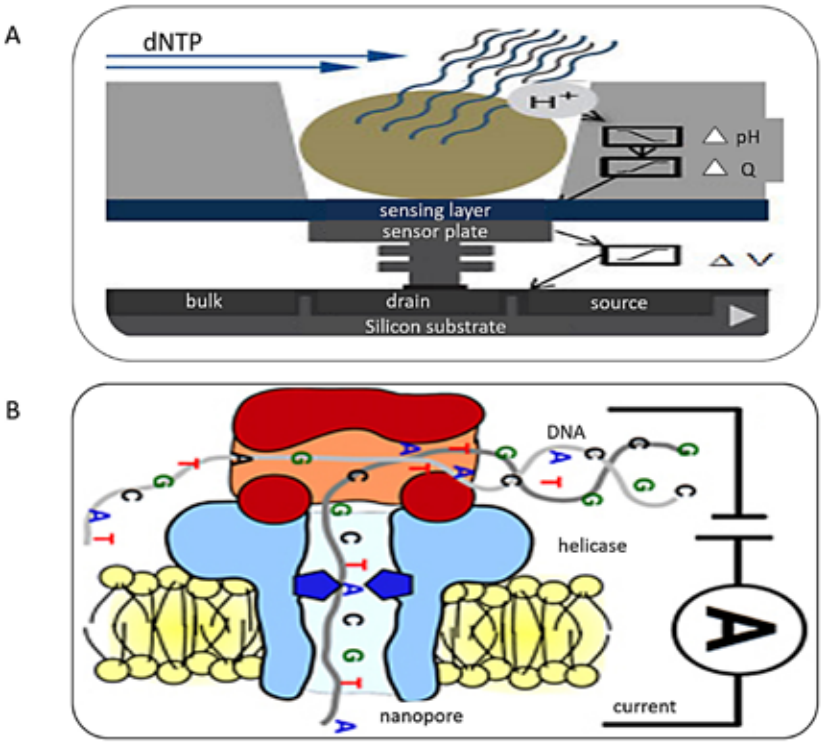

Fig. 2. Pictures briefing the chemistry and techniques used in the evolution of third or next generation DNA sequencing
(A)—Ion sequencing protons were released when growing DNA strands were incorporated by dNTP and change in pH in the well detected by the sensor and recorded as a nucleotide (Image source—en.genomics.cn); (B)—Oxford nanopore technique, nanopore incorporated into phospholipids bilayer and electric potential opposite side help DNA in translocation through the nanopore because of negative charge on it and membrane and ionic current is partially blocked to differentiate four nucleotides (Image source—author Steinbock, L. J., & Radenovic, A. (2015)

immobilization of the beads on a glass slide [8, 10] (Figure 2). The shortcoming of this method was the data analysis as the read length and depth was not the same as Illumina and created a problem in the assembly preparation [2].

Third-generation sequencing

The second-generation sequencing approach was incapable of handling: repetitive regions, to produce long reads, to recognize thousands of novel isoforms and gene fusion, therefore, more advanced and improved sequencing techniques were required [9] (Fig. 3). The third-generation techniques mainly focused on the single-molecule sequencing (SMS) first developed in the lab of Stephen Quake and the single-molecule real-time sequencing (SMRT) approach. Contrasting the SMS technology worked the same as the Illumina technique but without bridge amplification and this technique was relatively slow and expensive [14]. The basis of SMRT was the recognition of signals discharged using an array detection charge-coupled diode (CDD) in real-time, when they were incorporated, although, these two main techniques used DNA-polymerase and the terminal-phosphate-labelled nucleotide that allowed for sequencing long read length and short runtimes [1, 13].

The third-generation techniques were successful in the meantime, as they were capable of producing a huge amount of data at low cost and with less time as compared to the first generation sequencing [12]. During this phase, the setup of the sequencing machine reduced from giant size sequencing machine to a small cell phone size MinION sequencer (3rd Generation) and SmidION even smaller than a MinION (Figure 4).

The small size sequencer was designed in such a way that it can be connected into the laptop using the USB port and can be controlled by a Smartphone [23]. Among the seven types of nanopore sequencer, MinION and GridION worked with a biological nanopore in which the negatively charged DNA translocates through the nanopore placed into the phospholipids bilayer and when the positive electric potential introduced to the opposite side of the membrane translocation occurred and to allow the detection of four different nucleotides, the ionic current was partially blocked, leading to a reduction in the current, hence, DNA

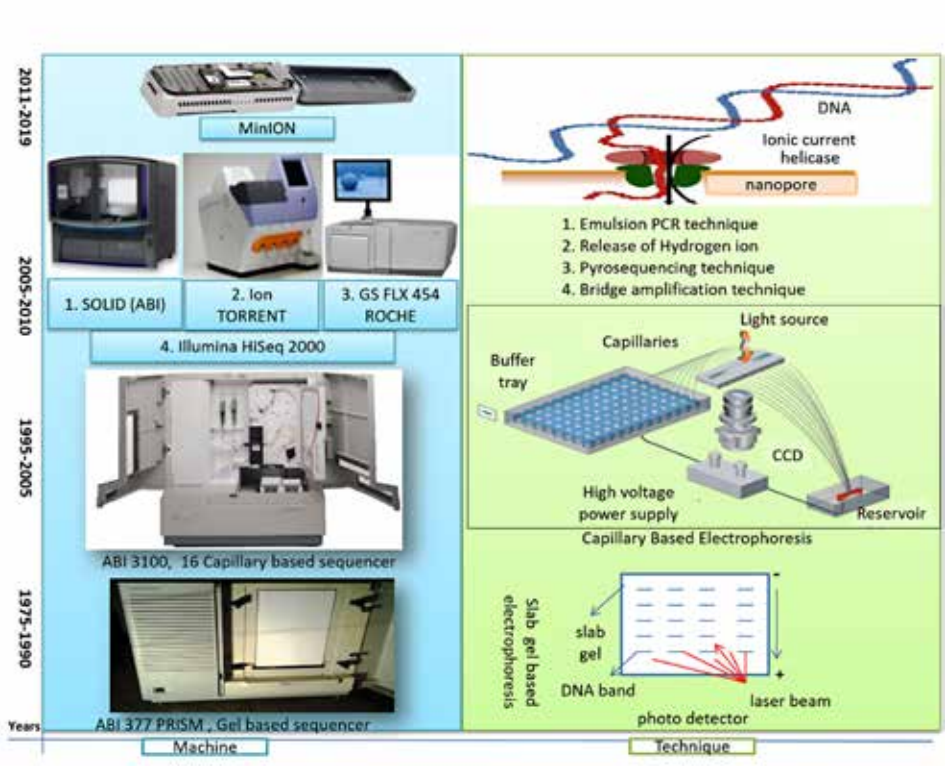

Fig. 3. Scheme depicting the evolution of sequencing machines and techniques

The development from bottom to top demonstrates the advancement in DNA sequencing and from giant size machine to small size sequencer. The Sanger chain termination methods using types of ddNTPs in 1975—2005; later, Illumina sequencing by synthesis techniques, Ion sequencing, pyrosequencing, sequencing by ligation, and the latest Oxford nanopore technique. The sources of images were from google images search and their respective official website

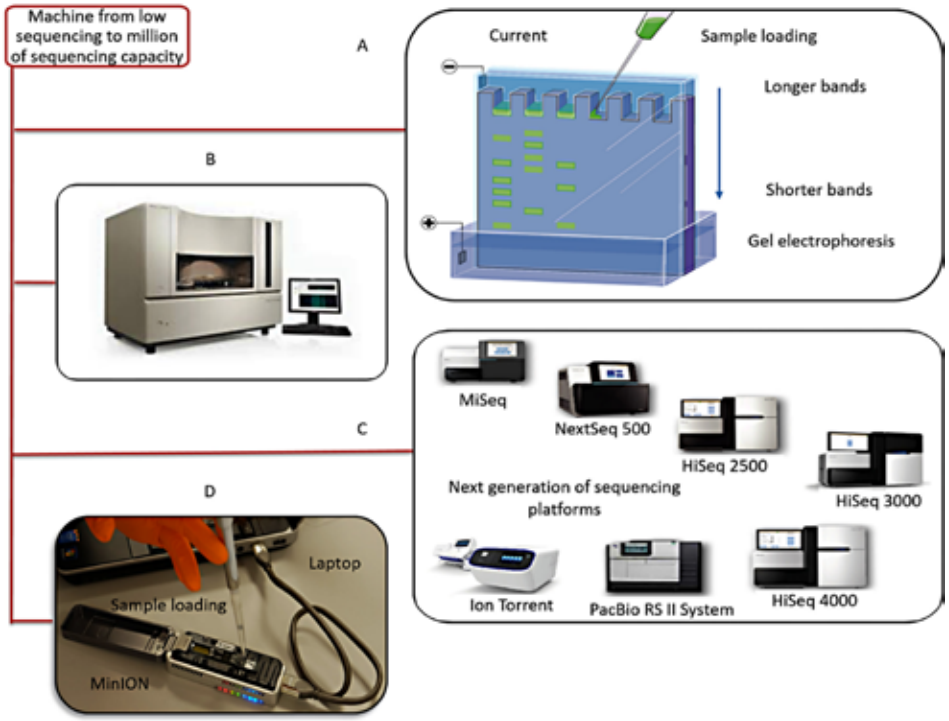

Fig. 4. Pictures explaining the evolution of DNA sequencing capacity from A low to D high

(A)—Slab gel-based DNA sequencing platform to separate labelled nucleotide based on their size (Image source—Smart.servier.com); (B)—First-generation automated Sanger sequencing machine with capillary method ABI 3730 sequencer; (C)—Advancement and second-generation Illumina sequencers with different in optic power and output capacity (Image source—base-asia.com); (D)—Third generation portable DNA sequencing instrument of Oxford Nanopore, MinION (Image source—Science-practise.com). Source: An original drawing with images sources from their respective origins

sequence inferred [22]. Soon, there was also a possibility to use non-biological, solid-state technology to design hybrid nanopores that might be capable of sequencing double-stranded DNA molecules [6].

We were aware that every technology had advantages and disadvantages; the potential drawbacks as compared to the second generation sequencing was the error rate that was over 90 % when the analysis was done using MinION on the lambda phage genome and amplicon of snake venom gland transcriptome [4]. Although, the advantage of compact size and compatibility to carry anywhere, Joshua Quick and Nicholas Loman successfully sequenced the Ebola virus genome in just 48 hours after the sample collection [3]. Nanopore technology was still in its initial phase of development in terms of accuracy and it will take time to be used in a wide range of application with high specificity.

CONCLUSIONS

It was desirable to achieve the highest possible accuracy in the field of sequencing because multiple factors can impact the final biological results. Therefore, there were scientists from the multidisciplinary fields worked together to refine these techniques. Over the last five-decades sequencing had progressed through sequencing the limited reads of pure RNA species to the whole set of eukaryotic genomes which was supported by the advancements in molecular biology, analytical chemistry and laser-induced fluoresce detection methods. Using DNA sequencing technology we are now able to understand the fundamental level properties that help to differentiate life. The overall evolution of sequencing techniques from using radioactive isotopes to changes in ionic current for the detection of nucleotide pattern and sequence opens up the possibility to sequence highly complex genomes with low cost, time and effort. The strength of DNA sequencing was that it can be applied to various omics and molecular diagnostic studies. It was reasonable that the future challenges will be aimed at achieving connectivity between data generated from the massively parallel sequencing and making repositories that can further help the researcher to get deeper biological insight. We believe that understanding the rich history of sequencing will establish a foundation for new sequencing techniques, as learning from previous factors leads to further progress.

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EFFECTS OF EARLY-LIFE ANTIBIOTICS ADMINISTRATION ON THE IMMUNE RESPONSE TO NEWCASTLE DISEASE LASOTA VACCINATION AND WEIGHT INDICES OF BROILER CHICKEN

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ABSTRACT

The administration of antibiotics to day old chicks as a means of prevention or treatment of suspected hatchery or farm-borne infections is common, especially in developing countries. This practice could contribute to a poor immune response following Newcastle disease (ND)-LaSota vaccinations, in addition to the sluggish growth in broiler chickens. This study was aimed at determining: the antibody titre to ND-LaSota vaccine, live weight, weight gain and feed conversion efficiency (FCE) of broiler chicken exposed early to gentamicin and doxycycline. One hundred, day-old broiler chicks were randomly assigned to four groups (n = 25). Group 1 served as a control, while groups 2 and 4 received gentamycin and doxycycline, respectively. The chicks in group 3 were treated with a combination of gentamicin and doxycycline (1 : 1). All drugs were administered via the drinking water from the 2nd to the 6th day of the chicks' life. On day 18, the birds received ND-LaSota vaccine intraocularly. At weekly intervals, the post-vaccination antibody titre, live weight and weight gain were determined. The feed conversion efficiency (FCE) of the different groups was calculated at the end of the experiments. The results showed that the NDV antibody titre of the antibiotic-treated groups did not differ significantly ($P < 0.05$) from that of the control. However, there was a significant ($P < 0.05$) increase in the live weight, weight gain and FCE of the control birds when compared to the antibiotic-treated groups.

Key words: antibody; doxycycline; gentamicin; titre; vaccination

INTRODUCTION

Several studies have shown that microbiota play a vital role in nutrient digestion and utilization [16, 17], as well as the modulation of innate [9, 12] and adaptive immune responses [11]. The colonization of the gut microbiota in young animals occurs simultaneously with the development of the gut tissues [5, 13, 15]; hence the disruption of gut microbiota as a result of early-life administration of

antibiotics could contribute to poor immune responses to vaccines as well as diminished production [14].

Antibiotics have continued to be used widely in the poultry industry, especially in developing countries [21]. In Nigeria for instance, broad-spectrum antibiotics are routinely given to day-old chicks during the first 5—7 days of their lives as a way of combating infections suspected to be hatchery-borne or already established in the brooding houses. This practice could be responsible for the observed poor antibody titre following Newcastle disease LaSota vaccination as well as sub-optimal levels of growth and productivity in poultry [3]. The possible outcomes of the above scenario include: outbreaks of Newcastle disease in vaccinated flocks, too frequent revaccination of birds against the disease, and increased cost of production on the part of poultry farmers [1, 3, 26].

The information on the effects of early-life administration of antibiotics on the immune response to vaccines, weight gain and overall performance of birds remains poor. However, studies in mice and human have shown that early-life modulation of microbiota colonization by antibiotics could result in the development of immunity-based disorders, such as asthma and allergy [25].

Surveys conducted in some parts of Nigeria have revealed that antibiotics mostly given to day-old chicks belong to the aminoglycosides and tetracyclines [4, 19], therefore in this study we selected gentamicin (a broad-spectrum aminoglycoside) and doxycycline (a member of the tetracycline class of antibiotics); both having broad-spectrum antimicrobial activity [10, 23].

MATERIALS AND METHODS

Experimental animals

One hundred unvaccinated day-old broiler chicks (Anak 2000®; Amo Farm Sieberer, Awe, Oyo State, Nigeria) were used for this study. The birds were raised intensively on deep litter, in standard poultry pens located at the Department of Veterinary Physiology and Pharmacology, Michael Okpara University of Agriculture, Umudike, Nigeria, between November and December of 2018. Using wood and wire gauze, the pen was uniformly divided into four partitions, each measuring 3.66 × 1.83 m.

Twenty-four hours prior to the chicks' arrival, the pen was heated to 33 °C and maintained at the same temperature throughout the first week. Subsequently, the brooding temperature was reduced by 3 °C weekly until the 4th week, when the source of heat was removed and the birds were maintained at an ambient temperature (22—25 °C). The birds were exposed to 24 hours of light throughout the experimental periods and fed ad libitum with commercial broiler starter (Top feed®, Nigeria). They were allowed free access to drinking water, except 1 hour prior to the vaccinations. The ethical standards governing the use of live animals for experiments as stipulated by Zimerman [31], Ward and Elsea [29] were strictly observed. The protocol for this study was approved by the College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike Research Ethics Committee, and was assigned the approval number: MOUAU/CVM/2018/003.

Experimental design

Upon arrival, the birds were randomly assigned to four groups (n = 25). Birds in group 1 served as a control and received plain water. Group 2 birds were given gentamicin (Gentamicin®; Greenlife Pharmaceuticals Ltd., Lagos, Nigeria) only, while birds in group 3 were treated with a combination of gentamicin and doxycycline (Doxygentavet®; Interchemie werken, Venray, Holland). The birds in group 4 received doxycycline only. All of the drugs were given at therapeutic doses, in drinking water for 5 consecutive days (days 2—6), following the manufactures' specifications. On day 18, the chicks were given ND-LaSota vaccine (ORNIPEST®; Bioveta, Czech Republic), via the intra-ocular route.

Sample collection

On the 18th day (prior to the ND-LaSota vaccinations) 2 ml of blood was collected from the wing vein of each of 4 birds randomly selected from each group. The blood was delivered into plain sample bottles, allowed to stand in a slanting position for 30 minutes, and then centrifuged at 2500 rpm. The harvested sera were stored in the freezer (–20 °C) until the time of use. Following this procedure, blood samples were collected from the birds and the sera were harvested at weekly intervals for four consecutive weeks. The birds that were sampled were properly identified so that no chick was bled more than once.

Preparation of 1 % chicken red blood cells (RBC)

Two millilitres of blood was collected from the wing

vein of 3 unvaccinated chickens housed separately from the experimental birds. The blood samples were delivered into test-tubes containing equivalent volumes of Alsever's anticoagulant and pooled. The sample was centrifuged at 1500 rpm for 5 minutes, the supernatant discarded, then replaced with phosphate buffered saline (PBS) and centrifuged again. This procedure was repeated thrice, then 1 % v/v suspension of the chicken RBC was achieved by adding 20 ml of PBS to 200 µl of washed RBC [22].

Preparation of virus antigen

The antigen was prepared from ND-LaSota vaccine (ORNIPEST®) by reconstituting a 200-dose vial of the vaccine in 2 ml of PBS, at pH 7.4 [3].

Determination of the haemagglutination titre of the antigen

This was done following the protocol of OIE [18], as described by Ali [2].

Phosphate buffered saline (25 µl) was placed into each well of a plastic V-bottomed 96-well microtitre plate.

The virus suspension (25 µl) was placed in the first well and twofold dilutions of 25 µl volumes of the virus suspension were made across the plate.

A further 25 µl of PBS was dispensed to each well, then 25 µl of the 1 % chicken RBC was added to each well. The plate was gently tapped for proper mixing, then held for 40 minutes at room temperature.

The hemagglutination was determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The highest dilution that gave a complete HA (no streaming) was taken as the HA titre, and represents 1 HA unit (HAU).

Hemagglutination inhibition (HI) test

Phosphate buffered saline (25 µl) was placed into each well of a plastic V-bottomed 96-well microtitre plate.

Serum (25 µl) was placed in the first well and twofold dilutions of 25 µl volumes of the serum were made across the plate.

Twenty-five microlitres of 4 HAU virus/antigen were added to each well and the plate was kept for 30 minutes at room temperature.

Twenty-five microlitres of 1 % (v/v) chicken RBCs were added to each well. After gentle mixing, the RBCs were allowed to settle for 40 minutes at room temperature.

The highest dilution of serum causing complete inhibition of 4 HAU of antigen was taken as the HI titre. The agglutination was determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs [18].

Live weight and feed conversion efficiency (FCE)

Upon the arrival of the birds, their live weights were determined using a digital balance. Subsequently, they were weighed every week. At the end of the 6-week experimental period, the FCE of each group of birds was calculated using the formula:

$$\text{FCE} = \frac{\text{weight gain}}{\text{feed intake}} \times 100$$

Statistical analysis

The data obtained were presented as means ± SEM and analysed using one-way analysis of variance (ANOVA) (SPSS software, version 20). The variant means were separated by the least significant difference (LSD) of the different groups. Significance was accepted at the level of $P < 0.05$.

RESULTS

The effect of early-life exposure of broiler chicken to gentamicin and doxycycline antibiotics on Newcastle disease virus antibody titre

The results showed that at week 0 (i. e. before ND-LaSota vaccinations), the ND antibody titre across the groups ranged between 1.75 ± 0.25 and 2.00 ± 0.48 [log2 geometric mean titre (GMT)]. It significantly increased and peaked at the 3rd week post-vaccination (5.25 ± 0.48—5.75 ± 0.25), after which it recorded a decline in all of the groups. However, throughout the experiments there were no significant ($P < 0.05$) differences between the antibody titre of the antibiotic-treated birds, when compared to those of the control (Table 1).

Effect of early-life gentamicin and doxycycline administration on the live weight, weight gain and feed conversion efficiency of broiler chickens

From the 4th to 6th week, there was a significant ($P < 0.05$) increase in the live weight of the control when compared to the antibiotics-treated groups (Fig. 1). There

Table 1. Newcastle disease virus antibody titre of broiler chicken exposed early to gentamicin and doxycycline antibiotics

Groups	Treatment	NDV antibody titre (log2 GMT)				
		Week 0	Week 1	Week 2	Week 3	Week 4
1	**Water**	1.75 ± 0.25	3.75 ± 0.25	5.50 ± 0.29	5.75 ± 0.25	4.75 ± 0.25
2	**Gentamicin**	1.75 ± 0.25	3.75 ± 0.29	5.50 ± 0.29	5.75 ± 0.25	4.50 ± 0.50
3	**Gentamicin + Doxycycline**	2.00 ± 0.48	4.00 ± 0.41	5.55 ± 0.25	5.25 ± 0.48	4.75 ± 0.25
4	**Doxycycline**	2.00 ± 0.48	3.50 ± 0.65	5.00 ± 0.41	5.50 ± 0.29	4.75 ± 0.25

Table 2. Effects of early-life gentamicin and doxycycline administration on the live weight and feed conversion efficiency of broiler chickens

Groups	Treatment	**Live weight** [kg] **Day 1**	**Feed intake** [kg])	**Live weight** [kg] **Day 42**	**Weight gain** [kg]	**Feed conversion efficiency** [%]
1	**Water**	0.041 ± 0.002	3.06	2.39 ± 0.14	2.349	76.7
2	**Gentamicin**	0.039 ± 0.001	3.09	2.03 ± 0.11*	1.991*	64.4
3	**Gentamicin + Doxycycline**	0.037 ± 0.001	3.09	2.09 ± 0.04*	2.053*	66.4
4	**Doxycycline**	0.040 ± 0.001	3.09	2.21 ± 0.07*	2.170*	70.2

*—P < 0.05 when compared with control

was also a significant (P < 0.05) increase in the weight gain of the control when compared to those of the antibiotics-treated birds. This was reflected in the 76.7 % FCE in the control group, against 64.4, 66.4 and 70.2 % FCE in the groups that received gentamicin alone, gentamicin + doxycycline and doxycycline alone, respectively (Table 2).

DISCUSSION

The haemagglutination inhibition test is the method of choice for the assessment of the immune response to the ND vaccines in birds [2, 22]; therefore it was employed in this study for the determination of the antibody titre following the ND-LaSota vaccinations. The low NDV antibody titre recorded at week 0 (before the LaSota vaccinations) was a reflection of waning maternal antibodies in the chicks [7, 24]. The significant increases in antibody titre (> log23.0) in all of the groups 2 weeks post-vaccination revealed that the ND-LaSota vaccine triggered positive (protective) immune responses to the Newcastle disease virus in the chicks [3,18].

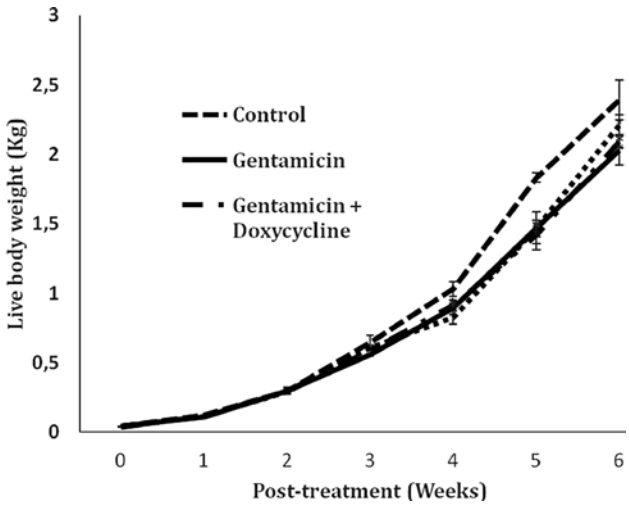

Fig. 1. Effect of early-life gentamicin and doxycycline on the live weight of broiler chickens
* P < 0.05 when compared with control

The non-significant difference (P > 0.05) in NDV antibody titre between the control and the other test groups demonstrated that early-life (day 2—6) administration of gentamicin and doxycycline to broiler chickens did not

of the gut microbiota between the termination of antibiotics administration (day 6) and the ND-LaSota vaccinations (day 18). Simon et al., [27] reported the recovery of the faecal microbiota composition of antibiotic-treated birds 2 weeks after cessation of the antibiotic administration. In our research, the antibiotics schedule (days 2—6) was aimed at mimicking common poultry production practice (in Nigeria) of brooding young chicks with broad spectrum antibiotics within their first week of life. The ND-LaSota vaccine was not administered until day 18 in order to allow for the decay of the maternal antibodies which would have interfered with the vaccine [24].

The significant increase in live weight, weight gain, and feed conversion efficiency of the control group compared with the antibiotic-treated groups may have resulted from the additional metabolic functions which the microbiome provided for the host chicken [20]. These functions include: nutrient absorption and utilization, fermentation of non-digestible dietary fibre, synthesis of bile acids, and scavenging of free radicals [6, 8, 16, 28].

CONCLUSIONS

We concluded that the exposure of broilers to gentamicin and doxycycline within their first week of life did not modulate immune response to the Newcastle disease LaSota vaccination carried out 12 days after terminating the antibiotics administration. However, the live weight, weight gain and feed conversion efficiency of the antibiotics-treated birds were significantly lower than those of the control.

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