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Proposed scheme for isolation and identification of *Clostridium perfringens* and *Clostridium perfringens*-like organisms

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ABDEL SALAM (I.S.), EL SANOUSI (S.M.). Description d'un protocole pour l'isolement et l'identification de *Clostridium perfringens* et des bactéries apparentées. *Revue Élev. Méd. vét. Pays trop.*, 1991, **44** (2) : 153-158

Les propriétés de 220 souches de *Clostridium perfringens* et des bactéries assimilées ont été étudiées. Les auteurs décrivent le protocole mis au point pour leur isolement et leur identification. Il est fondé sur la présence ou l'absence d'une enzyme, la lécithinase, sur l'hémolyse synergique avec la toxine de *Streptococcus* groupe B et l'inhibition de ces souches en présence des antisérums appropriés et leur réaction en milieu "lactose, gélatine, nitrate, mobilité (LGNM)" accompagné de la fermentation de certains sucres. *Mots clés* : *Clostridium perfringens* - Bactérie apparentée - Analyse microbiologique - Identification - Soudan.

INTRODUCTION

The conventional biochemical and serological tests that are routinely used for isolation, identification and confirmation of *Clostridium perfringens (C. perfringens)* and *C. perfringens*-like organisms are inadequate to identify all strains satisfactorily. The identification of *C. perfringens*-like organisms *per se* poses a problem. This is because some strains are unable to produce lecithinase or they are false lecithinase producers. Moreover, some strains of *C. perfringens* and *C. perfringens*-like organisms do not react synergestically with the ß-toxin of *Streptococcus* groupe B (3) while others, though reacting synergestically, do not react with commercially available specific antisera (5). Therefore, it is thought necessary to work out a scheme that can allow the identification of *C. perfringens* and *C. perfringens*-like organisms and this is the purpose of the present article.

MATERIALS AND METHODS

Strains

Two hundred and twenty strains of *C. perfringens* and *C. perfringens*-like organisms, isolated from the digestive tract of broilers of the Lohman German breed and Sudanese local breeds were used in this study. Broilers apparently healthy as well as those suffering from diarrhoea were used. A few strains were also isolated from litter and poultry feed. *Clostridium perfringens* NCTC 8238 (Hobbs) type 2, *C. barati* NCIB 10652, *C. beijerinckii* NCIB 9362, *C. absonum* FT/230 (Hobbs), *C. sardiniensis* 240680 (Beerens) and *C. barati* ATCC 27638 were used as reference strains.

Media

SHAHIDI and FERGUSON Perfringens Agar (SFPA) and Lactose Motility Agar (LMA) media were prepared according to SAHIDI and FERGUSON (17). Sugars used for study of fermentation were prepared according to HOLDEMAN and MOORE (10). The biochemical tests were conducted according to HAUSCHILD *et al.* (7), while the serological tests which included the lecithinase and synergestic haemolysis tests were prepared according to McCLUNG and TOABE (12) and GUBASH (3), respectively.

Methods

Black colonies on SFPA were subcultured on blood agars. Robertson's Cooked Meat Media were inoculated with pure colonies. Biochemical tests including sugar fermentation test were conducted. The organims were then tested for lecithinase production and inhibition with antisera (Wellcome, England).

False-positive lecithinase producers coupled with strains that failed to produce lecithinase were tested for their synergistic haemolysis reaction (3). Strains that reacted synergistically with ß-toxin of *streptococcus*, but failed to be initiated with specific antisera together with strains that completely failed to react synergistically with the ß-toxin were subjected to the lactose gelatin nitrate motility (LGNM) test and gelatinase detection. Moreover, the different shapes of the synergistic haemolysis that did not react with antisera were studied and recorded. The gelatin test was performed. The reactions of the gelatinase-possessers on the LGMN were recorded.

Non-gelatinase-possessers that fermented lactose, reduced nitrate and were non-motile were tested for their ability to ferment sugars. Aesculin hydrolysis was also performed. The by-products of liquid cultures were analysed for acetic and butyric acids and butanol presence using a Pye Unicam 104 series Gas Chromatograph (England).

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TABLE I** Properties of 220 strains of* **Clostridium perfringens** *and* **Clostridium perfringens-like organisms isolated from poultry-digestive tracts.*

Test	Degree	No. of strains	Test	Degree	No. of strains	Test	Degree	No. of strains
Haemolysis			C.M.M.			Lecithinase production		
	Strong	192		Saccharolytic	124		+	182
	Weak	7		non-saccharolytic	96		–	38
	- non	21						
Catalase	+	0	Growth at 10 °C	+	0	Lecithinase inhibited by antisera		
	–	220		–	220		–	142
Indole	+	193	15 °C	+	15		–	40
	–	27		–	205			
Nitrate	+	183	20-48 °C	+	220	Synergestic haemolysis		
	–	37		–	0		+	156
Gelatin	Strong	35	50 °C	+	168		–	64
	Moderate	131		–	52			
	Weak	48	52 °C	+	32	Synergestic haemolysis inhibited by antisera		
	–	06		–	188			
							+	111
							–	45

TABLE II** Sugar fermentation by 220 strains of* **C. perfringens** *and* **C. perfringens-like organisms.*

	Strong	Moderate	Weak	Trace	Negative
Glucose	29	67	68	56	00
Arabinose	06	18	71	34	90
Salicin	03	30	26	36	125
Raffinose	02	09	39	48	122
Lactose	02	34	113	31	40
Sucrose	13	73	58	41	35
Mannitol	02	11	23	33	151
Maltose	09	83	54	33	41
Sorbitol	00	09	27	36	148
Rhamnose	00	04	22	23	171
Xylose	02	04	77	32	105
Trehalose	11	87	47	18	57
Inositol	03	35	32	27	123

RESULTS

The biochemical properties of the 220 strains, Naglers'reactions, synergestic haemolysis and the gelatin liquefaction results are shown in table I. The sugar fermentation results are shown in table II. The suggested scheme is illustrated in figure 1.

Any black colony on SFPA was suspected to be due to a *C. perfringens* or *C. perfringens*-like organism. Strains that produced an opalescence inhibited by antisera were considered as *C. perfringens*. Strains which produced opalescence not inhibited with antisera together with strains that reacted synergestically with the ß-toxin of *streptococcus* which was inhibited with antisera were also considered as *C. perfringens*. Conversely those strains whose synergestic haemolysis was not inhibited by antisera and which showed the buller-shape types of haemolysis were identified as *C. barati*. Similar strains which produced the "small" size (2-5 mm) synergestic haemolysis and which was inhibited only with high concentrations of antisera were also identified as *C. barati*. Strains that did not behave like the previous ones in the synergestic haemolysis test, but which were capable of liquefying gelatin, fermenting lactose, reducing nitrate and which were non-motile were considered as *C. beijerinckii*. Strains which did not liquefy gelatin and which were capable of fermenting raffinose, but which did not produce lecithinase were considered as *C. sardiniensis*. This is in contrast with SNEATH (21). Strains that failed to liquefy gelatin, but which fermented cellobiose and galactose, gave variable results on ribose, melibiose and trehalose, failed to ferment melizitose, raffinose, rhamnose and failed to hydrolyse aesculin, produced acetic acid, butyric acid with butanol as a by-product were regarded as *C. barati* (15). Strains which fermented sucrose, liquefied

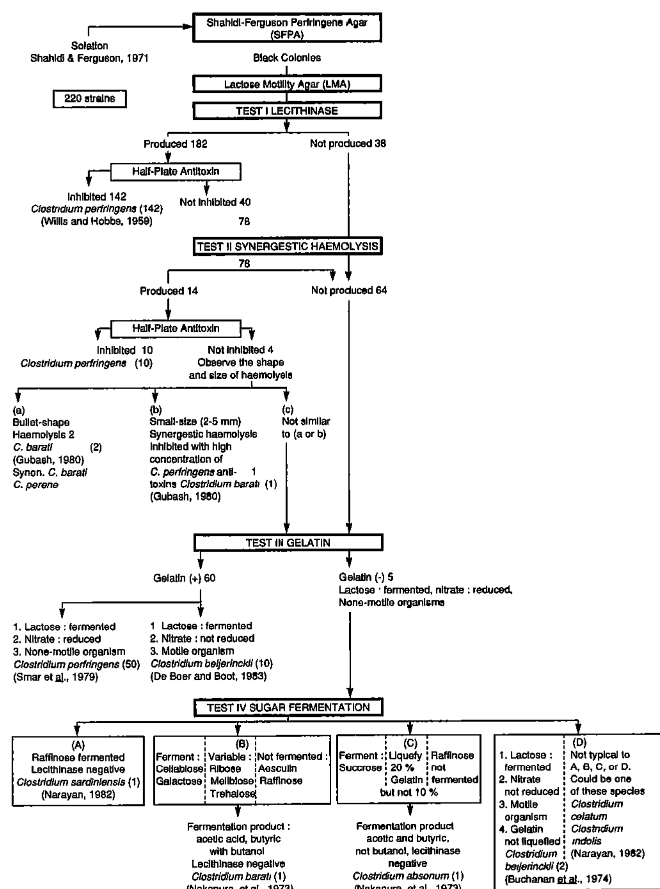

Fig. 1 : Proposed scheme for isolation and identification of Clostridium perfringens *and* Clostridium perfringens*-like organisms (figures in parenthesis are the identified species).*

2 % but not 10 % gelatin and failed to ferment raffinose with acetic and butyric acids without butanol as a by-product were considered as *C. absonum*. Strains that failed to liquefy gelatin, but fermented lactose, failed to reduce nitrate and were motile were regarded as *C. beijerinckii* (1). Strains that did not behave like the previous non-gelatin liquefyers were regarded as *C. celatum, Inflabilis indolis* (Syn *C. indolis*) or *Inflabilis lacustris* (16).

According to this scheme, the 220 strains were classified as follows : *Clostridium perfringens* 202 (142 produced opalescence ; 10 did not produce opalescence, but reacted synergestically with *streptococcus* and 50, did not produce opalescence, but liquefied gelatin and were non-motile), *C. barati* (identical to *C. perenne,* syn. *C. paraperfringens*, 4 strains ; three produced opalescence which was not inhibited with antisera and gave specific shapes of synergestic haemolysis ; one did not produce opalescence, did not liquefy gelatin and exhibited reactions on carbohydrates) ; *Clostridium beijerinckii* 12 (10 did not produce opalescence and liquefy gelatin ; two neither produced opalescence nor liquefied gelatin and exhibited a specific reaction on LGMN ; *Clostridium sardiniensis* 1 (failed to produce opalescence and liquefy gelatin, but fermented raffinose) ; *Clostridium absonum* 1 (failed to produce opalescence, liquefied 2 % but not 10 % gelatin, fermented raffinose, with acetic and butyric acids without butanol as a by-product.

DISCUSSION

Among the 220 strains tested for lecithinase production, 182 organisms known as false-negative lecithinase producers were found to produce lecithinase, but only 142 strains produced lecithinase, that was inhibited with *C. perfringens* antitoxin. The other 40 strains were recorded as false-positive lecithinase producers. Hence this test was found capable of identifying only 64.6 % of the strains tested. This was attributed mainly to the presence of strains that produce false-positive and false-negative lecithinase reactions in this medium. Such findings could be due to the presence of non-lecithinase producer organisms known as false-negative lecithinase producers (14). On the other hand, it may be attributed to the presence of strains which are physiologically related to *C. perfringens*. These strains include *C. barati* and *C. perfringens*-like strains. Such strains were reported to produce lecithinase reactions which were only inhibited with high concentrations of *C. perfringens* antitoxins. Hence these strains are termed as "false-positive" lecithinase producers. These findings are in keeping with those of NAKAMURA *et al.* (14) and GUBASH (3). HANDFORD (4) reported the presence of "false-positive" lecithinase reactions due to *C. bifermentans* and other clostridial spp. that reduce sulphide and produce lecithinase reaction in egg-yolk agar medium. These false-positive reactions which were not inhibited by *C. perfringens* antitoxin may also result from a shortage in the spectrum of the antisera used. Many workers reported that antisera are useless for identification of all *C. perfringens* strains (6, 20, 16 and 2). On the other hand, the lecithinase-negative isolates which were not identified by the Nagler's test were confirmed to be *C. perfringens* by use of other tests, *i.e.* the synergistic and LGNM test. The failure of these strains to produce lecithinase reaction could be due to the production of small amounts of toxins or to their weakness. Accordingly such strains were unable to produce a detectable lecithinase reaction. This phenomenon was exhibited by some strains of *C. perfringens*, *C. perfringens*-like organisms and *C. barati*. The presence of lecithinase-negative strains was reported by several workers (5, 10, 11, 12 and 20). It is interesting to note that these lecithinase-negative strains were reported to cause food-poisoning outbreaks (18, 19 and 20). These strains were previously designated as *C. plagarum* after HOLDEMAN and MOORE (10) and BUCHANAN *et al.* (1). NAKAMURA *et al.* (14) studied the properties of these strains in comparison with *C. perfringens*. They observed that these orga-

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nisms exhibit a great resemblance with *C. perfringens*, the only difference being that they are lecithinase-negative. They considered only these strains as "lecithinase-negative" variants of *C. perfringens*. They also recommended that the name *C. plagarum* should be cancelled and that the organims should be regarded as *C. perfringens*. In this study, the production of the lecithinase enzyme by some strains of *C. perfringens* and its absence in the growth media by other strains is reflected on other reactions produced by *C. perfringens* strains such as the mouse lethality and haemolysis reactions. One strain produced small amounts of a-toxins. It also exhibited weak lecithinase reactions and failed to produce haemolysis of sheep blood cells. Moreover, this strain was also non-lethal for mice. In contrast, strong lecithinase producers were found to be strongly haemolytic and highly lethal for mice.

Such findings confirm the association of a-toxin with haemolysis, lecithinase production and toxicity. Similar observations were previously reported (11).

When using the synergestic haemolysis and synergistic haemolysis and synergestic haemolysis half-plate antitoxin, 70.9 % of the tested strains were confirmed to be *C. perfringens*. Such findings disagree with those of GUBASH (3), who reported that 96.8 % of *C. perfringens* strains could be easily identified by such tests. Less than 1 % of the tested strains gave a false-positive reaction. This result is similar to the findings of HANSEN and ELLIOT (5). This false-positive reactions produced by some isolates were mainly due to *C. barati* and *C. perfringens*-like strains. These strains were found to produce different patterns of haemolysis. These include the buller-shape and the small-size (3-5 mm) synergestic lytic reactions. The former reaction was not affected by *C. perfringens* antitoxins, while the latter, *i.e.* small size, required higher concentrations of *C. perfringens* antitoxin to be inhibited. Other types of haemolysis which were not affected by *C. perfringens* antitoxin were also observed. The lower degree of affinity exhibited by these organisms to *C. perfringens* anti-toxins was reported by other workers (3, 14). The test was reported to differentiate only between *C. perfringens* and *C. barati*, but not between other *C. perfringens*-like strains, *i.e. C. absonum, C. sardiniensis, C. beijerinckii, C. celatum, C. perenne, C. indolis* and *C. lacustris*. This is because *C. barati* produces a characteristic reaction in the synergestic haemolysis test. These findings are similar to those of GUBASH (3) who described this test as a presumptive method for identification of *C. perfringens, C. paraperfringens* and other *Clostridial* spp.

The different patterns of haemolysis reported to be produced by some strains of *C. perfringens*-like organisms require further studies. HANSEN and ELLIOT (5) raised the question of the behaviour of lecithinase-negative strains in the synergestic tests. When such strains were tested, 14 (36.8 %) out of 38 strains were found to be able to produce synergestic reactions, of which 10 were inhibited by *C. perfringens* antitoxin and hence confirmed as *C. perfringens*. This test was found to be more efficient than the Nagler's test, and was capable of identifying 156 strains out of the 220 tested. Two of the remaining four strains gave the bullet-shape type of haemolysis and one strain gave the small-size type of haemolysis and all were considered as *C. barati*. The fourth strain reacted differently to the two groups. Sixty-four strains still remained unidentified.

Moreover, the problem of differentiating between *C. perfringens*-like organisms was not solved. MEAD, PAEZDE-LEON and ADAM (13) proposed the enzyme assay for differentiation of *C. perfringens*-like strains. These findings confirmed that the conventional methods described by various workers for the isolation and identification of *C. perfringens* were unable to give a prompt answer for all the strains recovered in this study. It was found that neither the Nagler's nor the synergestic haemolysis tests coupled with antisera alone were able to identify all the tested strains. Accordingly, the tests described by HAUSCHILD, HILSHEIMER and GRIFFITH (9), HAUSCHILD *et al.* (8), HAUSCHILD *et al.* (7) and SMART *et al.* (20) were used for confirmation and identification of the remaining 64 strains. The lactose fermentation, gelatin liquefaction, nitrate reduction and motility tests (LGNM) were done. Among the 64 strains, 40 were false-positive and 24 false-negative lecithinase producers. Of the 40 strains which produced a lecithinase not inhibited by antisera, 32 strains were reported to be LGNM positive. Hence, they were regarded as *C. perfringens*. Of the remaining eight strains seven were found unable to reduce nitrate and demonstrate positive motility, therefore according to DE BOER and BOOT (2), these strains were regarded and *C. beijerinckii*. One strain was unable to liquefy gelatin and to reduce nitrate but was motile. This strain was also regard as *C. beijerinckii* according to BUCHANAN *et al.* (1). The 24 strains that failed to produce lecithinase or synergestic haemolysis were tested similarly with the LGNM test. Eighteen strains found to be LGNM positive were classified as *C. perfringens* (20). Five strains were motile and failed to reduce nitrate and were hence considered as *C. beijerinckii* (2). One motile strain was found unable to reduce nitrate or liquefy gelatin and was thus identified as *C. beijerinckii* (1).

In conclusion, using the LGNM test, the 64 strains unidentified with serological tests were classified as follows : fifty strains were diagnosed as *C. perfringens* according to HAUSCHILD, HILSHEIMER and GRIFFITH (9), HAUSCHILD *et al.* (7), HAUSCHILD *et al.* (8) and SMART *et al.* (20). Ten strains were recorded as *C. beijerinckii* according to DE BOER and BOOT (2). The remaining five strains, which were gelatin-negative, were distributed as *C. sardiniensis, C. barati, C. absonum* and *C. beijerinckii* (1).

The scheme used in this study was found able to identify all the 220 strains of *C. perfringens* and *C. perfringens*-like strains. These findings encourage us to propose the use of this scheme for our routine diagnosis.

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ABDEL SALAM (I.S.), EL SANOUSI (S.M.). Proposed scheme for isolation and identification of *Clostridium perfringens* and *Clostriudium perfringens*-like organisms. *Revue Élev. Méd. vét. Pays trop.*, 1991, **44** (2) : 153-158

The properties of 220 strains of *Clostridium perfringens* and *Clostridium perfringens*-like organisms were studied. A scheme was designed for the identification of these strains. The scheme was based on the presence/or absence of lecithinase enzyme, synergestic haemolysis with *Streptococcus* group B toxin, their inhibition with appropriate antisera and reaction in the lactose gelatin nitrate motility test (LGNM) with the fermentation of a few sugars. *Key words* : *Clostridium perfringens* - Like organism - Microbiological analysis - Identification - Sudan.

ABDEL SALAM (I.S.), EL SANOUSI (S.M.). Descripcion de un protocolo para el aislamiento y la identificación de *Clostridium perfringens* y otras bacterias cercanas. *Revue Élev. Méd. vét. Pays trop.*, 1991, **44** (2) : 153-158

Se estudiaron las propiedades de 220 cepas de *Clostridium perfringens* y otras bacterias similares. Se describe el protocolo utilizado para el aislamiento e identificación. Este se basa en la presencia o ausencia de la enzima lecitinasa, sobre la hemólisis sinérgica con la toxina del *Streptococcus* grupo B, así como la inhibición de éstas cepas en presencia de antisueros apropriados y su acción en un medio de lactosa, gelatina, nitrato mobil (LGNM) junto con la fermentación de algunos azúcares. *Palabras claves* : *Clostridium perfringens* - Bacteria similares - Análisis microbiológico - Identificación - Sudán.

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