Table of Contents

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FORMATION CONTINUE - ARTICLES DE SYNTHÈSE

Les norovirus bovins, virus entériques méconnus dans l'espèce bovine

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RESUME : Les norovirus appartiennent à la famille des *Caliciviridae*. Ils ont été mis en évidence dans de nombreuses espèces animales : bovins, porcins, murins et très récemment chez un félin, le lionceau (*Panthera leo*). Chez l'homme, ils sont reconnus depuis plusieurs années comme des agents majeurs de gastroentérite humaine de cause non bactérienne, épidémique ou sporadique, souvent d'origine alimentaire. Malgré leur relative faible pathogénicité, ces virus, de par leurs différentes caractéristiques, constituent un problème de santé publique, surtout dans les communautés humaines. Les norovirus mis en évidence chez des animaux de production, en particulier chez les bovins, pourraient représenter dans ce contexte un risque zoonotique.

Dans cet article, les différentes données concernant les norovirus bovins sont exposées à la lumière de celles rassemblées pour les norovirus humains. Les hypothèses et questions, notamment d'ordre zoonotique, restant en suspens à leur sujet sont également envisagées.

INTRODUCTION

L'impact réel des virus dans les cas sporadiques ou épidémiques de gastroentérites alimentaires humaines était encore récemment sous-évalué par rapport aux agents étiologiques d'origine bactérienne. Les progrès réalisés au niveau du diagnostic moléculaire au cours de ces trois dernières décennies ont cependant permis à la médecine de repousser les limites du diagnostic et de l'étendre à de nombreux virus concernés (notamment les norovirus, rotavirus, virus de l'hépatite A et virus de l'hépatite E). Parmi les causes virales de gastroentérite, les norovirus (NoV) ont pris une importance toute particulière. Isolés dans un premier temps dans l'espèce humaine, chez qui ils constituent désormais une des causes majoritaires des épidémies de gastroentérites (Kohli *et al*., 2005 ; Widdowson *et al.*, 2005), les NoV ont également été mis en évidence chez différentes espèces animales. Cette extension du spectre d'hôte des NoV à différentes espèces animales, notamment des espèces appartenant à notre chaîne alimentaire (bovins, porcins) (Bridger *et al*., 1984 ; Sugieda *et al*., 1998), en a rendu leur étude plus importante encore ne serait-ce que pour les secteurs concernés par la manipulation des aliments ainsi que par les processus de fabrication alimentaire. Cette revue propose de faire le point sur les différentes données de littérature à propos des norovirus bovins (BoNoV), celles-ci étant traitées comparativement aux données en provenance de l'étude des norovirus humains (HuNoV). Les BoNoV pourraient constituer des pathogènes nouveaux à prendre en compte dans les élevages.

LES NOROVIRUS BOVINS

Classification

Les NoV, auxquels appartiennent les BoNoV, font partie de la famille virale des *Caliciviridae*. Au sein de cette famille, l'*International Committee on Taxonomy of Viruses* (ICTV) a décrit

quatre genres : les genres *Norovirus* (ex-*Norwalk-like virus*), *Sapovirus* (ex-*Sapporo-like virus*), *Lagovirus* et *Vesivirus* (Green *et al.*, 2000 ; Mayo, 2002). Les virus appartenant au genre *Norovirus*, dont le prototype est le virus de Norwalk (NV), ont été associés à des symptômes de gastroentérite aiguë chez l'homme (Green *et al.*, 2001). Les NoV affectent également plusieurs espèces d'animaux tels les bovins, les porcins et les murins (Sugieda *et al.*, 1998 ; Liu *et al.*, 1999a ; Karst *et al.*, 2003). Ils ont été tout récemment mis en évidence chez un félidé, plus précisément chez un lionceau en captivité (Martella *et al.*, 2007). Initialement basée sur la morphologie des virus, la classification s'est rapidement fondée sur les données de biologie moléculaire. Actuellement, cinq génogroupes (G) sont décrits au sein du genre *Norovirus* sur base de l'analyse des séquences nucléotidiques et en acides aminés d'une région de la séquence codant pour la polymérase virale dans le cadre ouvert de lecture ou *Open Reading Frame* 1 (ORF 1) (Ando *et al.*, 2000 ; Green *et al.*, 2000). Cependant, si un consensus a été obtenu au niveau des genres et des génogroupes, aucune consigne officielle n'a été mentionnée pour une classification plus précise en dessous du niveau du génogroupe. Cette classification s'articule habituellement sur l'analyse génétique de différentes courtes séquences de la région de la capside. Zheng et collaborateurs (2006) ont proposé une classification sur base de l'analyse complète de la séquence en acide aminés (aa) de la protéine de capside car ce système de classification est peu affecté par le phénomène de recombinaison. Ce phénomène, observé chez de nombreux virus (Awadalla, 2003 ; Thiry *et al.*, 2005), siège chez les NoV dans les gènes codant pour la polymérase virale et la capside (Han *et al.*, 2004 ; Oliver *et al.*, 2004 ; Bull *et al.*, 2005 ; Rohayem *et al.*, 2005). En analysant les séquences codant pour la protéine de capside de 164 souches, 29 sous-groupes (génotypes) ont été décrits dans le genre *Norovirus* : 8 dans le G I, 17 dans le G II, 2 dans le G III, 1 dans les G IV et V (les souches de référence pour chacun des génotypes ainsi que leur numéro d'accès dans *Genbank* sont repris dans le tableau I). Les souches de NoV infectant l'homme (HuNoV) se retrouvent ainsi majoritairement classées dans les deux premiers génogroupes (I et II) ; les quelques souches humaines restantes appartiennent au G IV. Les souches de BoNoV se classent dans le G III où elles ont été subdivisées en deux génotypes classiquement dénommés Jena (G III.1) et Newbury 2 (G III.2). Ces souches sont phylogénétiquement plus proches des norovirus humains de G I (Zheng *et al.*, 2006).

Tableau I : Souche de référence et numéro d'accession dans *Genbank* pour chaque génotype décrit dans les cinq génogroupes de norovirus. Le cryptogramme représentant la souche reprend les informations suivantes : l'espèce infectée par le norovirus (Bo : bovin, Hu : humain, Mu : murin), le lieu où la souche a été identifiée, l'année d'identification et le pays d'origine (CA : Canada, DE : Allemagne, JP : Japon, NL : Pays-Bas, SR : Arabie Saoudite, UK : Grande Bretagne, US : Etats-Unis).

Génogroupe	Souche de référence	Numéro d'accession dans Genbank	Génotype
I	Hu/NoV/Norwalk/8FIIa/1968/US	M87661	1
	Hu/NoV/Southampton/1991/UK	L07418	2
	Hu/NoV/Desert Shield 395/1990/SR	U04469	3
	Hu/NoV/Chiba 407/1987/JP	AB022679	4
	Hu/NoV/Musgrove/1989/UK	AJ277614	5
	Hu/NoV/Hesse/1997/DE	AF093797	6
	Hu/NoV/Winchester/1994/UK	AJ277609	7
	Hu/NoV/WUGI/2000/JP	AB081723	8
II	Hu/NoV/Hawaii/1971/US	U07611	1
	Hu/NoV/Melksham/1994/UK	X81879	2
	Hu/NoV/Toronto 24/1991/CA	U02030	3
	Hu/NoV/Bristol/1993/UK	X76716	4
	Hu/NoV/Hillington/1990/UK	AJ277607	5
	Hu/NoV/Seacroft/1990/UK	AJ277620	6
	Hu/NoV/Leeds/1990/UK	AJ277608	7
	Hu/NoV/Amsterdam/1998/NL	AF195848	8
	Hu/NoV/Idaho Falls/378/1996/US	AY054299	9
	Hu/NoV/Erfurt/546/2000/DE	AF427118	10
	Hu/NoV/SaitamaT29GII/2001/JP	AB112221	11
	Hu/NoV/Aichi 76-96/Chitta/1996/JP	AB032758	12
	Hu/NoV/M7/1999/US	AY130761	13
	Hu/NoV/Kashiwa47/2000/JP	AB078334	14
	Hu/NoV/SaitamaKU80AGII/1999/JP	AB058582	15
	Hu/NoV/SaitamaT53GII/2002/JP	AB112260	16
	Hu/NoV/CS-E1/2002/US	AY502009	17
III	Bo/NoV/Jena/1980/DE	AJ011099	1
	Bo/NoV/Newbury 2/1976/UK	AF097917	2
IV	Hu/NoV/Alphatron/98/1998/NL	AF195847	1
V	Mu/NoV/MNV1/2002/US	NC008311	1

Historique

En 1929, Zahorsky introduisit pour la première fois le terme de *Winter Vomiting Disease* pour décrire une maladie atteignant principalement des enfants, bien que quelques adultes aient été affectés et dont les principales manifestations cliniques étaient des vomissements, parfois exacerbés. À ces vomissements s'ajoutèrent chez certains patients des crampes abdominales et de la diarrhée ; ces symptômes disparaissaient rapidement et un rétablissement complet était de règle. La maladie se révèla très infectieuse (Zahorsky, 1929). Durant les quarante années suivantes, plusieurs épisodes similaires furent rapportés dans la littérature et suspectés être imputables au même agent (Goodall, 1954 ; Cumming et McEvedy, 1969).

En octobre 1968, une épidémie de gastroentérite toucha une école fondamentale dans la localité de Norwalk en Ohio, Etats-Unis. Durant une période de deux jours, la moitié des élèves et des professeurs fut touchée. Il y eut un taux d'attaque secondaire de 32 % à travers les contacts que les personnes infectées eurent avec leur entourage. Les symptômes développés concordaient avec ceux décrits plus tôt pour la *Winter Vomiting Disease.* Les examens diagnostiques de laboratoire pratiqués à l'époque sur les échantillons ne donnèrent tout d'abord aucun résultat jusqu'à ce que Kapikian et collaborateurs (1972) mettent en évidence l'agent responsable par immuno-microscopie électronique. Ce virus et d'autres identifiés par la suite furent rassemblés sous la dénomination *Norwalk-like viruses*. Les avancées technologiques dans le domaine de la biologie moléculaire et, de ce fait, les progrès dans le diagnostic de ces virus allaient cependant révéler l'amplitude du problème de santé publique qu'ils représentaient (Kapikian, 2000).

Des virus présentant des caractéristiques morphologiques très proches de celles des calicivirus furent ensuite découverts dans les matières fécales de bovins. La souche Newbury 2 a été isolée dans un échantillon de matières fécales d'un veau atteint de gastroentérite aiguë en Grande-Bretagne en 1978. Administré à des veaux gnotobiotiques, cet isolat reproduisait des symptômes de gastroentérite. Il ne pouvait être multiplié en culture de cellules (Woode et Bridger, 1978). L'agent fut caractérisé et observé en microscopie électronique par Bridger et collaborateurs (1984). La souche de Jena fut identifiée pour la première fois en Allemagne, également chez des veaux atteints de gastroentérite (Gunther *et al.*, 1984 ; Gunther et Otto, 1987). Le séquençage partiel ou total de leur génome a relié génétiquement ces deux souches au genre *Norovirus* (Liu *et al.*, 1999a ; Oliver *et al.*, 2006a).

Caractéristiques morphologiques et biologiques

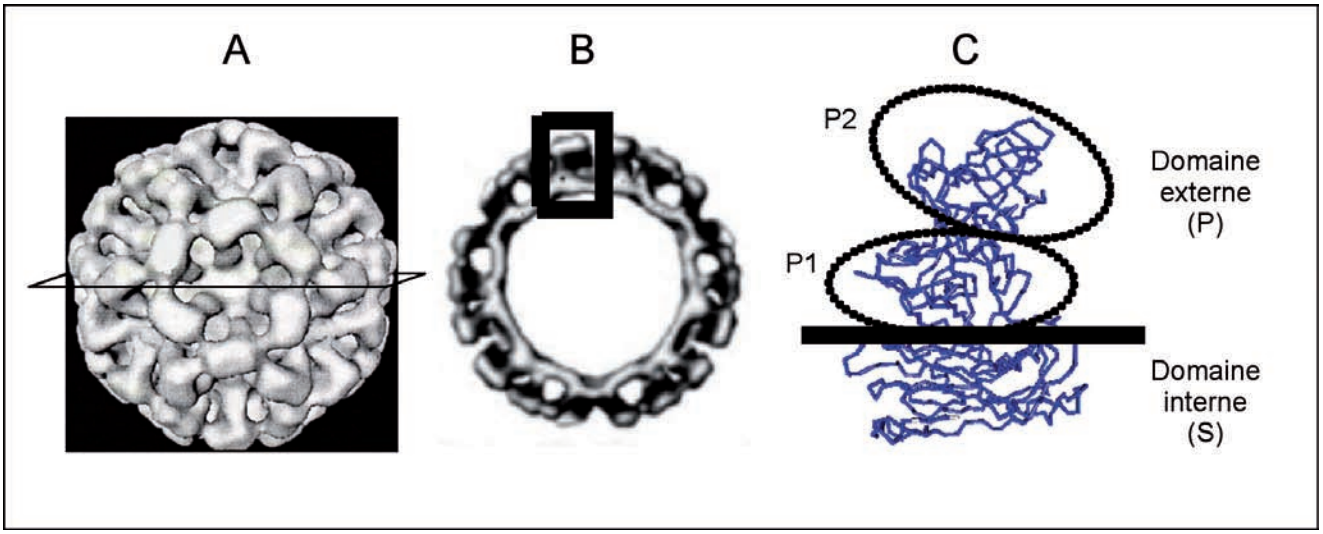
Les calicivirus auxquels appartiennent les BoNoV ont hérité leur nom des 32 dépressions caractéristiques en forme de calice présentes en surface de leur capside et qui peuvent être observées en microscopie électronique. Non enveloppés, les BoNoV présentent une capside d'un diamètre compris entre 27 et 38 nm ; cette capside est composée de 180 copies d'une protéine unique appelée protéine virale 1 (*viral protein* 1, VP1). Ces protéines s'assemblent en dimères pour former des capsomères qui s'organisent de façon à donner une capside de symétrie icosaédrique de T=3 (figure 1A) (Prasad *et al.*, 1994 ; Prasad *et al.*, 1999). Une densité de flottaison en chlorure de césium de 1,33 à 1,41 g/ml leur est associée (Green *et al.*, 2001). Ces virus se révèlent résistants dans le milieu extérieur et cela même en conditions très défavorables de pH ou de température (Dolin *et al.*, 1972).

La multiplication des norovirus *in vitro* en culture de cellules reste encore, à ce jour, fastidieuse, à l'exception notable du norovirus murin (MuNoV) (Duizer *et al.*, 2004b ; Wobus *et al.*, 2004). Très récemment, un système de culture tridimensionnel soumis à des flux de milieu a pu être mis au point pour la réplication *in vitro* des HuNoV et ouvre des perspectives intéressantes tant pour les HuNoV que pour les BoNoV (Straub *et al.*, 2007).

Organisation du génome et virologie moléculaire

Le génome des BoNoV est un ARN monocaténaire de polarité posi-

Figure 1 : *Structure de la capside d'un norovirus* ***A :*** *représentation tridimensionnelle de la surface de la capside d'un norovirus, résolution en cryo-microscopie électronique à 22 Å ;* ***B :*** *section tangentielle de la capside d'un norovirus ;* ***C :*** *Structure tertiaire évaluée par Swiss Model (accessible sur internet via l'adresse http://swissmodel.expasy.org) de la protéine de capside d'un norovirus bovin isolé en laboratoire dans le service de Virologie et Pathologie des Maladies virales animales de la Faculté de Médecine vétérinaire de Liège (souche B309).*



Deux monomères s'assemblent pour former un dimère et 90 dimères rentrent dans la composition de l'entièreté de la capside. Le domaine S (partie N-terminale de VP1) intervient dans la constitution de la structure icosaédrique interne de la capside et le domaine P (partie C terminale) forme les arches des calices en surface de la capside. Le sous-domaine P1 est modérément conservé entre les différentes souches tandis que le sous-domaine P2, le plus exposé au système immunitaire de l'hôte, est très variable.

tive (Green *et al.*, 2001). Par analogie avec ce qui a été décrit pour d'autres calicivirus ainsi que pour le MuNoV (Burroughs et Brown, 1978 ; Daughenbaugh *et al.*, 2006), une protéine VPg pourrait être liée à l'extrémité 5' de l'acide nucléique viral. Trois ORF sont mentionnés dans l'organisation du génome des NoV (Jiang *et al.*, 1993). À l'extrémité 5', l'ORF 1 code pour une polyprotéine d'environ 1740 aa (195 kDa) qui sera par la suite clivée par la protéinase virale et par son précurseur pour donner les protéines non structurales du virus (Liu *et al.*, 1996 ; Liu *et al.*, 1999b ; Belliot *et al.*, 2003 ; Scheffler *et al.*, 2007). L'ORF 2 encode l'unique protéine de capside (VP1) tandis que le produit de l'ORF 3, la protéine virale 2 (*viral protein* 2, VP2), est une petite protéine mineure structurale (figure 2). La majorité des données moléculaires émanent de l'étude de souches humaines. Cependant, les BoNoV présentant la même organisation génomique (Liu *et al.*, 1999a ; Oliver *et al.*, 2006a) que celle des souches humaines, des fonctions similaires pour leurs protéines sont logiquement attendues. De l'extrémité 5' vers l'extrémité 3' sont décrites les protéines suivantes :

P48

In vitro, cette protéine se colocalise avec des protéines cellulaires de l'appareil de Golgi et serait responsable de sa dislocation lorsqu'elle est surexprimée (Fernandez-Vega *et al.*, 2004). Un rôle d'interférence dans le transport et le trafic protéique intracellulaire associé aux membranes lui est attribué (Ettayebi et Hardy, 2003).

NTPase

Cette protéine présente une activité de liaison et d'hydrolyse des nucléotides triphosphates. Elle partage des motifs avec la protéine 2C des picornavirus et plus généralement avec les hélicases de la superfamille 3 (Pfister et Wimmer, 2001).

P22

Très peu de données sont disponibles dans la littérature au sujet du rôle de cette protéine. Les similitudes constatées entre NoV et picornavirus au niveau génétique et, particulièrement une position semblable à celle de la protéine 3A de ces derniers permettent de postuler une localisation des complexes de réplication associée aux membranes intracellulaires et donc un impact sur le trafic intracellulaire associé à ces membranes (Doedens *et al.*, 1997). De telles propriétés sont également rapportées pour p30, l'équivalent de p22 chez le calicivirus félin, un vésivirus (Green *et al.*, 2002).

VPg

Elle serait la protéine liée de manière covalente en 5' à l'ARN viral. Cette liaison a seulement été mise en évidence expérimentalement pour certains calicivirus animaux. Cette protéine pourrait jouer un rôle dans l'initiation de la traduction et le recrutement des ribosomes étant donné qu'*a contrario* des ARNm cellulaires, l'ARN viral des calicivirus ne possède ni coiffe ni site d'entrée ribosomal interne (Herbert *et al.*, 1997 ; Gutierrez-Escolano *et al.*, 2000 ; Daughenbaugh *et al.*, 2003). Ces hypothèses ont été confirmées pour le MuNoV (Daughenbaugh *et al.*, 2006) mais restent à déterminer pour les autres norovirus.

3CLpro

Cette protéine exerce une activité de type protéase et est impliquée dans le clivage de la polyprotéine initiale (Liu *et al.*, 1996). Ses propriétés enzymatiques ont été caractérisées (Someya *et al.*, 2005). Par son activité de clivage de la protéine liée à la queue polyadénylée, elle est responsable d'une inhibition de la synthèse protéique cellulaire et donc jouerait un rôle dans la régulation de l'expression génique tant virale que cellulaire (Kuyumcu-Martinez *et al.*, 2004).

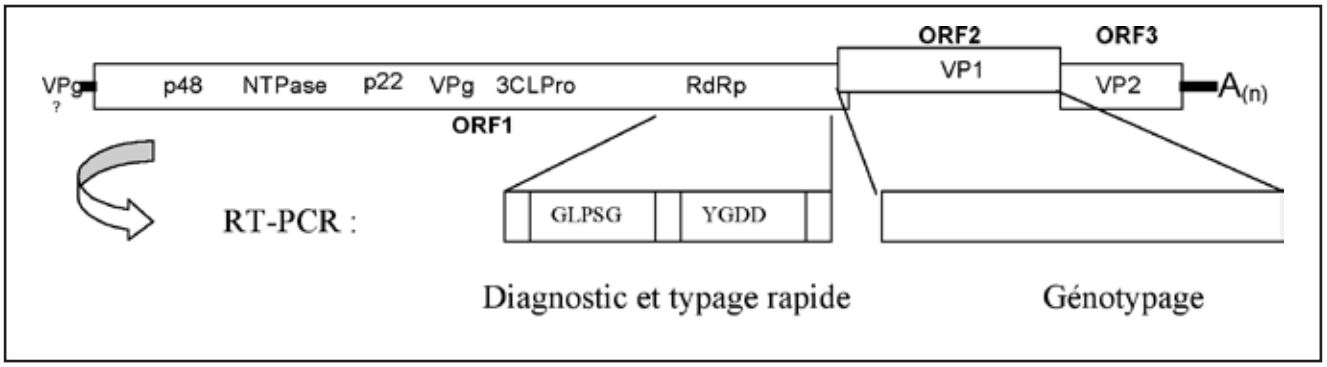
ARN polymérase ARN dépendante virale

Elle partage les mêmes caractéristiques structurales et catalytiques que les polymérases virales des autres virus à ARN de polarité positive (Ng *et al.*, 2004).

VP1

VP1 est l'unique protéine rentrant dans la composition de la capside virale. Elle possède une masse

Figure 2 : *Organisation génomique des norovirus et régions génomiques classiquement ciblées pour le diagnostic des infections à norovirus par RT PCR (motifs GLPSG et YGDD).*



Le séquençage du produit de l'amplification peut permettre de classer la souche au sein d'un génogroupe. Le génotypage nécessite l'amplification et le séquençage de régions génomiques partielles codant pour la protéine de capside VP1 ou de la séquence complète de celle ci. VPg : protéine virale potentiellement liée au génome ; p48 : protéine virale 48 ; NTPase : nucléotidetriphosphatase ; 3CLPro : protéase virale 3C-like ; RdRp : ARN polymérase virale ARN dépendante ; VP1 : protéine majeure structurale (protéine de capside) ; VP2 : protéine mineure structurale ; ORF : cadre de lecture ouvert.

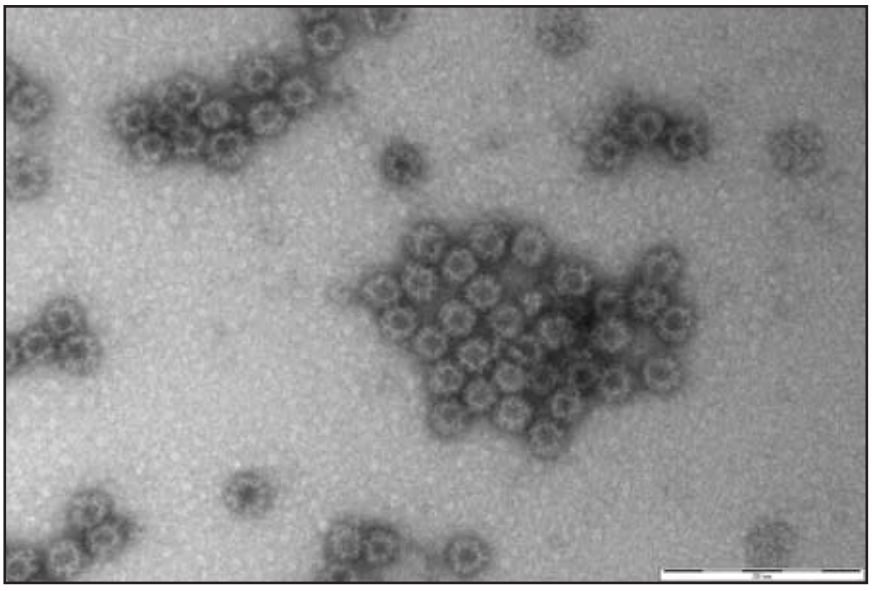
moléculaire d'environ 56 kDa pour plus ou moins 530 aa suivant les souches de NoV. La protéine de capside des BoNoV est légèrement plus courte que celles des HuNoV : 520, 523, 531 et 536 aa respectivement pour les souches de Jena (G III.1, BoNoV), Newbury 2 (G III.2, BoNoV), Norwalk (G I.1, HuNoV) et Hawaï (G II.1, HuNoV) (Jiang *et al.*, 1993 ; Lew *et al.*, 1994 ; Liu *et al.*, 1999a ; Oliver *et al.*, 2006a). La structure primaire de VP1 se compose de deux domaines. Le domaine S (pour *shell domain*), très conservé, contient les éléments essentiels pour la formation de la structure icosaédrique de la capside. Le domaine P (pour *protruding domain*) forme les arches des calices en surface, supporte les déterminants antigéniques et est subdivisé en deux sous-domaines : P1, modérément conservé, et P2, région hypervariable impliquée dans les interactions avec la cellule (figure 1) (Prasad *et al.*, 1999 ; Lochridge *et al.*, 2005).

Plusieurs études ont montré que la VP1 de HuNoV interagissait avec des oligosaccharides liés à ceux du complexe majeur d'histocompatibilité ABO et de Lewis (Hutson *et al.*, 2002 ; Marionneau *et al.*, 2002 ; Huang *et al.*, 2003 ; Tan *et al.*, 2004 ; Tan et Jiang, 2005a ; 2005b) et les reconnaissait comme récepteurs, conférant à l'infection par les HuNoV une certaine notion de sensibilité/résistance génétique. Les individus de phénotype sécréteur, c'est-à-dire génétiquement déficients pour la 1,2-α-fucosyltransférase, une enzyme impliquée dans les voies de biosynthèse de ces oligosaccharides, semblent prédisposés à l'infection par la souche de Norwalk (Marionneau *et al.*, 2002 ; Hutson *et al.*, 2005 ; Larsson *et al.*, 2006). Les épidémies de gastroentérites causées par les norovirus sont fréquemment associées à la consommation de fruits de mer. Il a été démontré que les mollusques bivalves peuvent concentrer les NoV dans leurs tissus digestifs par l'expression des oligosaccharides impliqués comme récepteurs pour les souches humaines (Le Guyader *et al.*, 2006).

À l'heure actuelle, les récepteurs des NoV animaux ne sont pas encore caractérisés. Cependant, les résultats d'une étude récente ont démontré la réplication d'un HuNoV chez des porcs exprimant des oligosaccharides similaires à ceux du système ABO et de Lewis en surface des cellules de l'intestin grêle (Cheetham *et al.*, 2006 ; 2007). On sait depuis longtemps que l'espèce porcine exprime en surface de ses cellules des antigènes tissulaires fort semblables à ceux décrits pour l'espèce humaine (Yamamoto et Yamamoto, 2001). D'autre part, ce type d'antigène tissulaire serait largement exprimé en surface des cellules de nombreuses autres espèces animales avec, bien entendu, des variations plus ou moins sensibles suivant les espèces (Marionneau *et al.*, 2001). Il est donc envisageable que de telles structures soient également impliquées comme récepteurs cellulaires pour le BoNoV. Tout récemment, la réplication d'une souche humaine avec induction de lésions et de signes cliniques d'entérite a également été montrée chez des veaux gnotobiotiques (Souza *et al.*, 2008).

Ces virus ne se répliquant que difficilement en culture de cellules, les scientifiques ont cherché à obtenir de différentes manières des substituts pour leur étude. Parmi celles-ci, le système d'expression protéique en baculovirus a rapidement permis d'obtenir de tels substituts. Comme la structure de la capside des NoV en général et des BoNoV en particulier est relativement simple (une seule protéine structurale), les protéines VP1 exprimées en système baculovirus s'assemblent spontanément pour former des pseudoparticules virales (*virus-like particles*, VLPs) structurellement, morphologiquement et antigéniquement semblables aux norovirus d'origine (Jiang *et al.*, 1992b ; Deng *et al.*, 2003 ; Lochridge et Hardy, 2003 ; Han *et al.*, 2005) (figure 3). Ces VLPs constituent donc un outil d'étude adéquat pour ce genre de virus et leur immense potentiel a été rapidement perçu puisqu'elles permettent de disposer de grandes quantités antigéniques d'un virus que l'on ne peut produire *in vitro*. Ce potentiel a été principalement exploité dans les secteurs du diagnostic (comme matériel de base au développement d'outils de détection basés sur les réactions immunologiques tels que

Figure 3 : *Pseudoparticules virales (VLPs) produites par expression en système baculovirus de la protéine de capside d'une souche de norovirus bovin isolée en laboratoire dans le service de Virologie et Pathologie des Maladies virales animales de la Faculté de Médecine vétérinaire de Liège (souche B309).*



Les protéines produites se sont spontanément assemblées pour former des particules présentant une morphologie classique de calicivirus avec des structures en forme de calice et un diamètre compris entre 27 et 38 nm. Ces VLPs constituent morphologiquement et structurellement un excellent substitut d'étude des norovirus. Image en microscopie électronique après coloration à l'acétate d'uranyle réalisée par Jan Mast (CERVA).

les ELISAs) et de l'étude de l'immunité développée contre les NoV.

VP2

VP2 est une petite protéine structurale basique de 35 kDa (Glass *et al.*, 2000), présente à raison de 2 ou 3 molécules par virion (Prasad *et al.*, 1994), dont le rôle n'est pas encore clairement établi mais qui stabiliserait VP1 et la protégerait du désassemblage et de la dégradation (Bertolotti-Ciarlet *et al.*, 2003).

Pathogénie et aspects cliniques

Les NoV mis en évidence dans les différentes espèces animales partagent de nombreuses propriétés pathogéniques. La voie de transmission des NoV est habituellement oro-fécale (Woode et Bridger, 1978 ; Graham *et al.*, 1994). Elle peut être de trois types : d'individu à individu, par l'intermédiaire des aliments et de l'eau, et à partir de surfaces contaminées. Une très faible dose infectieuse (inférieure à 100 particules virales), une forte stabilité dans l'environnement (Rzezutka et Cook, 2004), une résistance relative à l'inactivation (Duizer *et al.*, 2004a) et une grande diversité des souches sont des caractéristiques accroissant le risque d'infection.

Chez l'homme, les symptômes apparaissent après une période d'incubation de 1 ou 2 jours. Ils sont en général légers et de courte durée (Rockx *et al.*, 2002) excepté chez les personnes immunodéprimées ou débilitées (Goller *et al.*, 2004 ; Mattner *et al.*, 2006). Ces symptômes consistent majoritairement en des vomissements, parfois incoercibles, accompagnés ou non de diarrhée ou de fièvre avec un taux d'attaque secondaire très élevé, spécialement dans les communautés telles les maisons de repos, hôpitaux, écoles et bateaux de croisière (Caul, 1996).

Une diarrhée non hémorragique bénigne et de l'anorexie sont également les signes cliniques observés chez des veaux gnotobiotiques infectés avec des BoNoV (Bridger *et al.*, 1984). La RT-PCR a permis de mettre en évidence une longue durée d'excrétion dans les matières fécales (Han *et al.*, 2005). La résistance dans le milieu extérieur et la propagation rapide dans les communautés des HuNoV, ajoutés aux modes d'élevage pratiqués dans les exploitations suggèrent un impact plus important qu'il n'y paraît pour les BoNoV, au moins au niveau zootechnique.

L'infection par les BoNoV se localise dans le tractus digestif et l'entérite se limite à l'intestin grêle (Woode et Bridger, 1978 ; Granzow et Schirrmeier, 1985). Les lésions anatomopathologiques sont particulièrement marquées dans la région moyenne du jéjunum (Gunther *et al.*, 1984 ; Gunther et Otto, 1987).

Réponse immunitaire

Jusqu'à présent, aucune étude *in vivo* n'a visé à caractériser en détail l'immunité développée contre les BoNoV et la plupart des données disponibles ont été obtenues en mettant à profit les VLPs de BoNoV, substituts acceptables pour ce type d'étude. Plusieurs auteurs ont en effet prouvé leur immunogénicité par voie systémique, orale (la voie classique d'infection par les NoV) et même intranasale, chez la souris (Jiang *et al.*, 1992a ; Ball *et al.*, 1998 ; Guerrero *et al.*, 2001) comme chez l'homme ou les bovins (Tacket *et al.*, 2003 ; Han *et al.*, 2006). Afin de compléter au mieux ce chapitre, les données de réponse immunitaire humaine et murine, plus nombreuses, seront également présentées.

Les études réalisées sur des volontaires humains ont indiqué l'établissement d'une immunité protectrice de courte durée (environ 6 mois), dirigée contre les virus homologues mais pas nécessairement étendue aux virus hétérologues. Elle était suivie d'une sensibilité renouvelée à l'infection (Johnson *et al.*, 1990 ; Matsui et Greenberg, 2000). De plus, les individus présentant des anticorps sériques préexistants ne sont pas nécessairement protégés contre l'infection (Graham *et al.*, 1994). La réponse spécifique en immunoglobulines A (IgA) sériques apparaît être une constante ; ces IgA n'induisent pas de protection croisée entre génogroupes (Erdman *et al.*, 1989 ; Lindesmith *et al.*, 2005). Des IgA à réactivité croisée entre les G I et II ont pourtant récemment été mises en évidence dans le lait de femmes allaitantes (Makita *et al.*, 2007). Le pic en IgM apparaît 2 semaines après l'infection. Il n'est pas restreint à l'infection primaire mais constitue plutôt un marqueur d'infection récente (Cukor *et al.*, 1982 ; Brinker *et al.*, 1999). Les titres en IgG ne résultent pas de l'exposition mais bien de l'infection (Lindesmith *et al.*, 2005). Les infections expérimentales de veaux ont donné les mêmes profils de réponse immunitaire avec les pics en IgG atteints 3 semaines après l'infection (Han *et al.*, 2005). Comme mentionné précédemment, c'est VP1 qui supporte les déterminants antigéniques de l'immunité protectrice. Des épitopes localisés dans le domaine S permettent d'obtenir des réactions croisées entre génogroupes (Yoda *et al.*, 2003 ; Batten *et al.*, 2006 ; Oliver *et al.*, 2006b). Ainsi, les génotypes Jena et Newbury 2 sont antigéniquement distincts mais partagent au moins un épitope commun localisé dans le domaine S de leur protéine de capside avec les HuNoV de G II.3 permettant d'obtenir des réactions croisées (Oliver *et al.*, 2006a).

L'immunité innée joue un rôle important dans le contrôle de l'infection par le MuNoV étant donné que l'immunité adaptative B- et T-dépendante n'est pas requise pour la protection. Une protéine de la famille des transducteurs et activateurs de la traduction du signal (STAT 1) est impliquée. Les interférons (IFNs), autres composants de l'immunité innée, sont également concernés puisque des souris délétées des récepteurs aux IFNs $\alpha\beta$ et γ sont plus sensibles à l'infection létale et que la multiplication virale est sensible aux IFNs (Karst *et al.*, 2003 ; Chang *et al.*, 2006). Les VLPs de BoNoV pourraient aussi être utilisées dans le développement d'un vaccin (Estes *et al.*, 2000 ; Tacket *et al.*, 2003 ; Han *et al.*, 2006).

Diagnostic

Les méthodes actuelles de diagnostic des NoV sont la microscopie électronique, les méthodes immunologiques et celles basées sur l'amplification et la détection de séquences génomiques (RT-PCR, qRT-PCR) (Atmar et Estes, 2001). La microscopie électronique, bien que restant une méthode de choix, est fastidieuse, coûteuse, peu sensible et nécessite une grande expérience. La sensibilité des méthodes immunologiques reste limitée par la grande diversité antigénique des souches de NoV. Enfin, le diagnostic moléculaire (figure 2) est le dernier à avoir été développé et a bénéficié du séquençage complet ou partiel de nombreuses souches (tableau I). Elle est la méthode la plus sensible et permet actuellement une approche quantitative qui restait jusqu'il y a peu impossible ou peu

précise. Ses inconvénients sont une constante mise à jour des sondes utilisées pour se trouver en adéquation avec les souches circulantes issues soit des mutations aléatoires et de la pression de sélection exercée par le système immunitaire, soit du phénomène de recombinaison. Jusqu'à présent aucune paire d'amorces universelles n'a pu être mise au point et celles utilisées pour le diagnostic de routine s'hybrident dans une région conservée du gène de la polymérase (Atmar et Estes, 2001 ; Smiley *et al.*, 2003 ; Vinje *et al.*, 2003 ; Ike *et al.*, 2007).

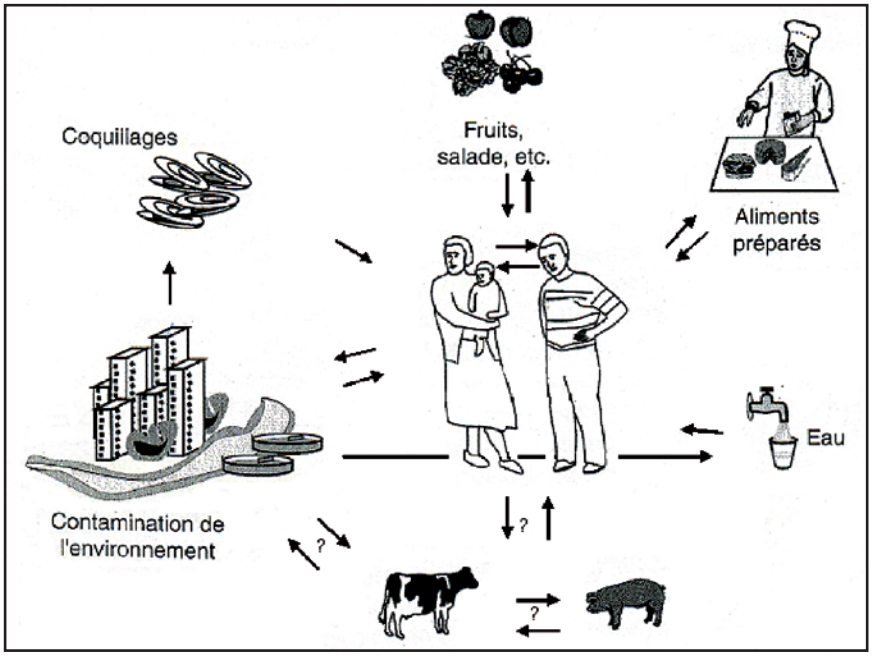
L'utilisation d'un contrôle interne permet d'augmenter la spécificité du test de diagnostic en diminuant la part des faux négatifs due à la présence d'inhibiteurs de RT-PCR (Scipioni *et al.*, 2008a).

La technologie des microdamiers est envisagée et il serait étonnant qu'elle ne soit pas mise au point dans un futur proche (Jaaskelainen et Maunula, 2006).

Distribution géographique

Les études épidémiologiques ont fréquemment démontré que les NoV sont largement répandus et que les infections sont courantes autant chez l'homme que chez les bovins, les porcins et les murins (Scipioni *et al.*, 2008b). À l'heure actuelle des séquences de BoNoV ont été mises en évidence par biologie moléculaire sur tous les continents habités mis à part l'Afrique. Pour ce dernier continent, il faut peut-être y voir tout simplement un biais de recherche. Les prototypes des deux génotypes décrits ont été mis en évidence dans le courant des années'80 ; le premier en Grande Bretagne (génotype 2, Newbury 2) et ensuite en Allemagne (génotype 1, Jena) (Bridger *et al.*, 1984 ; Gunther et Otto, 1987). Dans ce dernier pays, des BoNoV du génotype 2 ont aussi récemment été diagnostiqués par Ike et collaborateurs (2007). Par RT-PCR sur échantillons de matières fécales, des prévalences de 9 % (Deng *et al.*, 2003), 11 % (Milnes et *al*., 2007), 31,6 % chez des veaux et 4,2 % chez des vaches laitières (Van Der Poel *et al.*, 2003) ont été mises en évidence respectivement en Allemagne, en Grande Bretagne et aux Pays-Bas. La séroprévalence envers le génotype Jena est de 99 % chez des vaches laitières de Thuringe, de Hesse et de Bavière (Deng *et al.*, 2003). Des BoNoV ont été détectés dans des exploitations en Belgique (Scipioni *et al.*, 2004 ; Scipioni *et al.*, 2008a), où une forte séroprévalence apparente a pu également être démontrée (Mauroy *et al.*, 2007), aux Etats-Unis, dans le Michigan et le Wisconsin (Wise *et al.*, 2004), au Canada (Mattison *et al.*, 2007), au Vénézuela (Alcala *et al.*, 2003), en Corée du Sud (Park *et al.*, 2007) et en Nouvelle-Zélande (Wolf *et al.*, 2007).

Figure 4 : *Voies de transmission classiques des norovirus et risque zoonotique potentiel constitué par les bovins et les porcs en terme de portage, de réservoir, de transmission, de multiplication et d'opportunité de recombinaison des norovirus. Adapté de Loisy et collaborateurs (2004).*



DISCUSSION ET CONCLUSION

Les NoV sont largement répandus tant chez l'homme que chez les animaux de production. Chez les bovins, l'impact zootechnique des NoV n'est sûrement pas négligeable quoique peu évalué à l'heure actuelle, surtout en Belgique. De par leurs caractéristiques biologiques, les NoV bénéficient d'une relative stabilité environnementale et constituent donc un réel problème d'assainissement des lieux contaminés. Leur voie de transmission étant orofécale, une des sources communes d'infection chez l'homme est constituée par les aliments contaminés par des eaux d'effluents et ingérés crus comme les fruits de mer (China *et al.*, 2003). Chez les bovins, cette transmission via l'alimentation devrait être investiguée.

Bien que ce risque soit fort discuté selon les auteurs, la proximité génétique de certains NoV animaux avec les souches humaines pourrait faire craindre un risque zoonotique par leur présence dans les eaux d'effluents, surtout dans des pays comme la Belgique où se conjuguent densité humaine et concentration d'élevages. Récemment la multiplication et la pathogénicité de HuNoV a été montrée chez des porcs gnotobiotiques (Cheetham *et al.*, 2006). Il s'agit là du premier risque zoonotique clairement identifié et les souches porcines (PoNoV) sont d'ailleurs relativement proches des souches humaines au niveau génétique ; elles ont été classées dans le G II, groupe majoritairement constitué de souches humaines (Sugieda et Nakajima, 2002). Pour l'espèce bovine, aucune co-infection n'a été enregistrée jusqu'à ce jour et le récepteur cellulaire pour le BoNoV n'a pas encore été caractérisé. Si son récepteur devait montrer une relative proximité avec celui du HuNoV, alors l'espèce bovine tout comme l'espèce porcine pourrait jouer un rôle de réservoir pour les HuNoV. Des opportunités de recombinaison entre norovirus humains et animaux existeraient alors avec comme corollaire l'émergence et

la diffusion de nouvelles souches. La présence simultanée de NoV humains et animaux a été mise en évidence dans des mollusques (Costantini *et al.*, 2006). Plus récemment, de très courtes séquences se classant phylogénétiquement dans le G II.4 (génotype de souches humaines) ont été obtenues par RT-PCR à partir d'échantillons fécaux bovins (Mattison *et al.*, 2007). Ces éléments renforcent encore les craintes émises quant au risque zoonotique représenté par les souches de NoV animaux (figure 4).

De nombreuses études complémentaires sont nécessaires pour identifier toutes les composantes de ce risque. Ensuite, ce risque devra être caractérisé ; l'étude du récepteur cellulaire du BoNoV en est un des exemples. Des études épidémiologiques devront être réalisées dans l'espèce bovine afin d'y caractériser l'impact zootechnique et sur la santé du cheptel des BoNoV. D'une manière plus large, de telles études seraient également intéressantes non seulement dans les autres espèces de production (porcins, volaille), mais peut être aussi chez les animaux de compagnie.

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SUMMARY

Noroviruses belong to the *Caliciviridae* family. They have been identified in several species: bovine, porcine, murine and recently in a lion cub (*Panthera leo*). They are known since several years as major agents of foodborne non-bacterial human gastroenteritis, both epidemic and sporadic. Although they are characterised by a low pathogenicity, these viruses are a real threat for public health because their different properties, especially in human communities. In this context, noroviruses identified in production animals, in particular cattle, could represent a non negligible zoonotic risk.

This article deals with data concerning bovine noroviruses in the light of human noroviruses knowledge. Hypothesis and unanswered questions are also considered, in particular the ones relative to zoonotic risk.

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The reduction of murine norovirus 1, *B. fragilis* HSP40 infecting phage B40-8 and *E. coli* after a mild thermal pasteurization process of raspberry puree

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ABSTRACT

Pasteurization processes of raspberry puree are nowadays limited to short times and rather low temperatures to maintain flavor and nutritional quality. Norovirus (NoV) outbreaks associated with raspberries highlight the need to determine the survival of NoV on this type of soft fruit. Therefore, resistance of murine norovirus 1 (MNV-1), a surrogate for human NoV, *B. fragilis* HSP40 infecting phage B40-8, and *E. coli* towards mild pasteurization was tested. Raspberry puree heat treated at 65 °C for 30 s showed a 1.86, 2.77, and 3.89 log reduction of, respectively, MNV-1, *E. coli*, and B40-8. Heating at 75 °C for 15 s established a 2.81 log reduction of MNV-1 while a 3.44 and 3.61 log reduction of B40-8 and *E. coli* was observed. No supplementary lethal effect of holding the heat-treated raspberry puree at 4 °C overnight was noticed. B40-8 failed to be useful as a tool to monitor NoV inactivation during mild pasteurization processes. Moreover, <3 log reductions of MNV-1 were observed suggesting that upon high initial contamination load, infectious NoV particles may remain on mildly pasteurized raspberry puree.

1. Introduction

In many European countries, desserts based on raspberries are popular. However, the role of raspberries is well established as a source of Norovirus (NoV) outbreaks. Frozen raspberries mixed with fresh cheese, a raspberry drink and bakery products were reported to be the presumptive cause of several NoV outbreaks in France, Sweden and Denmark (Cotterelle et al., 2005; Korsager et al., 2005; Hjertqvist et al., 2006). NoV is characterized by a high attack rate resulting in outbreaks, where often hundreds of infected people are involved. In general, the illness associated with NoV infection is mild. Diarrhea, vomiting, nausea are typical symptoms, but severe illness and even death can occur in sensitive age groups such as young children and the elderly (Goller et al., 2004; Sakai et al., 2001). Fecally contaminated irrigation water or feces-based fertilizer applied on the field could contaminate raspberries with foodborne viruses. The berries are harvested by food pickers which may, by unhygienic handling, introduce NoV onto the raspberries (Carter, 2005). Raspberries are mostly processed into individually quick frozen (IQF) berries or to puree as intermediatory products. Raspberry puree can be further processed in the food chain to smoothies, fruit fillings, yoghurt, and jams or jellies. The production of puree implies a heat treatment to enhance the shelf life and to comply with microbiological regulations. Raspberry puree is in general pasteurized by heating at 88 °C for about 2 min (Sinha, 2006). This pasteurization process would be sufficient to inactivate NoV (Strazynski et al., 2002; Baert et al., 2008a; Buckow et al., 2008). However, because of the customer's demands for minimal processed foods in order to maintain nutritional and flavor aspects, the pasteurization processes of raspberries are mitigated and comprise nowadays a mild heating step of either 30 s at 65 °C or 15 s 75 °C. In order to validate the HACCP plan of raspberry processing companies, knowledge is needed into which extent this mild heat treatment warrants reduction of NoV and consequently may guarantee a microbiological safe product i.e. absence of the NoV hazard.

In the current study, the reduction of murine norovirus 1 (MNV-1) by these mild heat treatments was investigated. MNV-1 was used as a surrogate for human NoV because the human strains require a complex cell system to grow (Straub et al., 2007). MNV-1 is not pathogenic for humans but causes gastro-enteritis in mice. The pathogenesis in mice makes MNV-1 unacceptable to be used as an indicator outside a laboratory environment. According to the preferences of ideal indicators described by Busta et al. (2003), *B. fragilis* HSP40 infecting phages seemed to be promising to monitor NoV contamination. In addition, the effect upon the classical microbiological hygiene indicator *E. coli* was

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questioned. Standard pasteurization processes as mentioned by Sinha (2006) would be sufficient to inactivate *B. fragilis* phages and *E. coli* sufficiently (Kory and Booth, 1986; Ugarte-Romero et al., 2006). In this study, the potential of both micro-organisms to act as indicators for viral contamination of mildly pasteurized raspberry puree was investigated.

2. Methods

2.1. MNV-1 plaque assay

2.1.1. Cell culture

RAW 264.7 cells (kindly provided by Prof H. W. Virgin, Washington University School of Medicine, MO) were maintained in DMEM (Cellgro, Mediatech, Herndon, Virginia) containing 10% FBS (HyClone, Logan, Utah), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), 10 mM HEPES (Invitrogen), and 2 mM L-Glutamine (Invitrogen) and grown at 37 °C under 5% CO_2 atmosphere.

2.1.2. MNV-1 lysate

RAW 264.7 cells were infected with MNV-1.CW1, passage 5 (kindly provided by Prof H. W. Virgin) at MOI of 0.05 for 2 days. Two freeze/thaw cycles and low-speed centrifugation removed cellular debris from the virus lysate as described by Wobus et al. (2004). The supernatant (i.e. MNV-1 lysate) was taken and stored in aliquots at −75 °C. The number of MNV-1 plaque forming units (PFU)/ml was determined by plaque assay as described by Wobus et al. (2004).

2.2. B40-8 phage assay

2.2.1. Strains

Bacteriophage B40-8 and the host strain *B. fragilis* HSP40 were kindly provided by Dr. Muniesa and Dr. Jofre (University of Barcelona, Spain).

2.2.2. B40-8 lysate

B. fragilis HSP40 was grown in *Bacteroides* phage recovery medium (BPRM) as described by Tartera et al. (1992). The host strain grown until 2×10^8 CFU/ml was infected at a MOI of 0.1. After 16–20 h, 0.3 vol. of chloroform (VWR, Fontenay-sous-Bois, France) were added to the culture and left overnight at room temperature. The top layer was centrifuged (10 min, 4000 × *g*, 4 °C) and the supernatant was kept at 4 °C (i.e. B40-8 lysate). The number of B40-8 PFU/ml was determined by plaque assay as described by Araujo et al. (2001).

2.3. E. coli enumeration

E. coli (LMG 8223; BCCM™/LMG Culture Collection, Ghent, Belgium), was cultured in Tryptone Soy Broth (Oxoid, Hamphire, England) supplemented with yeast extract (Oxoid) at 37 °C. *E. coli* was enumerated by a pour plate count method using Coli ID agar (Biomerieux, Lyon, France) as a selective medium. Plates were incubated at 37 °C for 24 h and specific *E. coli* colonies were enumerated as specified by the product descriptions.

2.4. Pasteurization of raspberry puree inoculated with MNV-1 and B40-8

Deep-frozen raspberry puree (100% raspberries, pH 3.1, 9.2° Brix) was kindly provided by a local fruit processing company. Raspberries were harvested in Poland and Serbia by food pickers of local families. After sorting, second-class raspberries were mashed without prior washing step or heat treatment in order to obtain raspberry puree. Raspberry puree was subsequently frozen and transported to Belgium. In general, before raspberry puree, an intermediatory food product, is sold to customers i.e. other food business operators in the food chain, a mild heat treatment of the raspberry puree is performed by the fruit processing company in Belgium. In order to validate the mild thermal pasteurization processes applied, the reduction of MNV-1, a surrogate for human NoV and B40-8, a potential indicator for viral contamination, was investigated in the current study. Raspberry puree (10 g) was thawed at 4 °C and transferred to stomacher bags with filter compartment (full filter blender bag, FBAG-04, 190 mm × 300 mm, Novolab, Geraardsbergen, Belgium). The samples (10 g of raspberry puree) were settled in a warm-water bath until the temperature of interest (65 or 75 °C) was reached and remained stable inside the sample.

A virus stock solution was prepared from MNV-1 lysate and B40-8 lysate in PPS (1 g/L peptone (Oxoid) and 8.5 g/L NaCl (Sigma Aldrich, Steinham, Switzerland)) to obtain a final concentration of 6.20 ± 0.38 log PFU/ml of MNV-1 and 5.89 ± 0.11 log PFU/ml of B40-8. One ml of the virus stock solution was added and dispersed in 10 g raspberry puree. The inoculated raspberry puree was kept in a warm-water bath at 65 °C or 75 °C for, respectively, 30 s or 15 s. After the heat treatment, raspberry puree was immediately cooled down on ice. The virus extraction procedure was performed on all heat-treated samples after a maximum storage of 1 h on ice. As a positive control, raspberry puree was inoculated with 1 ml virus stock solution at room temperature (RT). Additionally, a negative control was included, i.e. raspberry puree without heat treatment and without inoculation.

2.5. Pasteurization of raspberry puree inoculated with E. coli

Besides MNV-1 and B40-8, the classical microbiological hygiene indicator *E. coli* was also included to validate the mild heat treatments. A 24 h fresh culture of *E. coli* was diluted in PPS until 6.07 ± 0.11 log CFU/ml.

Similar to the virus inoculation procedure, 1 ml of *E. coli* was dispersed in raspberry puree which was kept at the temperature of interest. Tenfold serial dilutions of each sample (10 g raspberry puree) were prepared in PPS and *E. coli* was enumerated (see Section 2.3).

2.6. MNV-1 and B40-8 virus extraction

The virus extraction procedure was performed as described previously (Baert et al., 2008b) with some modifications. Briefly, 30 ml of elution buffer (0.1 M Tris-HCl (Sigma, S-Louis, MO), 3% beef extract (Sigma), 0.05 M glycine (Sigma), pH 9.5) and 150 μl of Pectinase (Sigma) was added to raspberry samples in a stomacher bag with filter compartment. After 20 min shaking, the filtrate was taken and centrifuged (10,000 × *g*, 15 min, 4 °C). The pH of the supernatant was adjusted to 7.2–7.4. PEG 6000 (Sigma) and NaCl (Sigma Aldrich) were added to obtain a final concentration of 10% PEG-0.3 M NaCl. The samples were placed on a shaking platform (rotating at 120 rpm, HS 260, Staufen, Germany) overnight (4 °C). The next day the samples were centrifuged (10,000 × *g*, 30 min, 4 °C). The supernatant was discarded; subsequently the pellet was dissolved in 2 ml PBS (Cellgro). The samples were finally stored at −75 °C until they were used to determine the PFU/ml by MNV-1 plaque assay and by B40-8 phage assay.

2.7. Statistical analysis

Samples were tested in triplicate and experiments were repeated three times. Error bars represent the standard deviations. Reductions were calculated as log (N_t/N_0). N_t represents the number of MNV-1, B40-8 or *E. coli* after a heat treatment performed at 65 or 75 °C. N_0 represents the initial number of MNV-1, B40-8 or *E. coli* without heat treatment. Statistical analyses of the reductions obtained by the pasteurization experiments were performed by One-way ANOVA (Tukey HSD was used as Post Hoc test) with SPSS 15.0 for Windows (SPSS Inc., US). Significant differences were considered when $P<0.05$.

3. Results

3.1. Enumeration of MNV-1 and B40-8 in raspberry extracts

Interferences of the cell culture and MNV-1 plaque assay were noticed when 2 ml viral raspberry extracts were 10 times diluted. Therefore, MNV-1 was titrated from raspberry extracts which were at least 100 times diluted in DMEM. The detection limit of the MNV-1 assay was consequently raised to 400 PFU MNV-1/10 g raspberry puree. Tenfold diluted raspberry extracts did not interfere with the B40-8 phage assay resulting in a detection limit of 20 PFU B40-8/10 g raspberry puree.

3.2. The reduction of MNV-1, B40-8, and E. coli on raspberry puree after a pasteurization process at 65 and 75 °C

Fig. 1 depicts the number of MNV-1, B40-8, and *E. coli* on raspberry puree after two pasteurization processes. Inoculated raspberry puree with MNV-1 and B40-8 (controls) contained, respectively, 5.75 ± 0.34 log MNV-1 PFU/10 g and 5.52 ± 0.49 log B40-8 PFU/10 g. A pasteurization process at 65 °C for 30 s showed a 1.86 ± 0.32 log reduction of MNV-1, while a 3.89 ± 0.46 log reduction of B40-8 was observed. The heat treatment at 75 °C (15 s) resulted in a 2.81 ± 0.39 log reduction of MNV-1 and a 3.44 ± 0.56 log reduction of B40-8.

Inoculated raspberry puree without heat treatment counted 5.97 ± 0.04 log CFU/10 g *E. coli* (i.e. control). Heating at 65 °C for 30 s caused a 2.77 ± 0.44 log reduction of *E. coli*. The heat treatment at 75 °C for 15 s resulted in a 3.61 ± 0.48 log decline of *E. coli*.

Heat-treated raspberry puree was cooled down and stored on ice for maximum 1 h before the virus extraction procedure or *E. coli* enumeration was performed. No decline was observed when inoculated raspberry puree without heat treatment was stored on ice for 1 h (data not shown). The obtained reduction indicated, therefore, solely the effect of heating in a raspberry puree matrix. MNV-1, B40-8, and *E. coli* were not detected on the negative controls (raspberry puree without heat treatment and without inoculation).

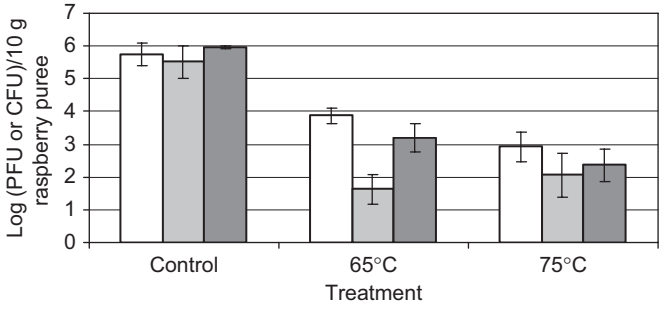

Fig. 1. The number of MNV-1, B40-8, and *E. coli* in raspberry puree after a mild pasteurization process. The log PFU/10 g raspberry puree of MNV-1 (white bars), B40-8 (gray bars) and the log CFU/10 g raspberry puree of *E. coli* (dark gray bars) are shown after 30 s 65 °C, 15 s 75 °C and without heat treatment (control). The error bars represent the standard deviations from 3 independent experiments including 3 replicates.

Table 1
Comparison of the number of MNV-1 and B40-8 directly determined on inoculated raspberry puree and after 24 h storage at 4 °C

	(log MNV-1_{direct})−(log MNV-$1_{24\,h}$)	(log B40-8_{direct})−(log B40-$8_{24\,h}$)
30 s at 65 °C	−0.0017	0.0089
15 s at 75 °C	0.00	−0.16
Control[a]	−0.20	1.84

[a] Control: raspberry puree without heat treatment.

The reduction observed for MNV-1, B40-8, and *E. coli* at 65 °C was all significant different from each other ($P<0.05$). *E. coli* and B40-8 showed a similar decline when heated at 75 °C ($P>0.05$), but differed significantly from MNV-1 ($P<0.05$). The heat treatment at 75 °C did not lead to a significant higher reduction for B40-8 compared to the heat treatment at 65 °C ($P>0.05$). In the case MNV-1 or *E. coli* were subjected to a heat treatment at 75 °C, higher reductions were established in comparison with 65 °C ($P<0.05$).

3.3. The reduction of MNV-1, B40-8 after pasteurization and 24 h storage at 4 °C

In the case MNV-1/B40-8 containing raspberry samples were pasteurized and subsequently stored at 4 °C for 24 h, no additional reduction in titer could be observed (Table 1). Control samples stored for 24 h, resulted in a decline of 1.84 log of B40-8 but no decrease of MNV-1 was noticed.

4. Discussion

The last decennia several foodborne outbreaks linked to frozen raspberries increased and the majority was caused by NoV. The strong association of NoV with raspberries can be supported by several explanations; (i) raspberries have a considerable risk to come into contact with fecal contamination on the field, which enhances their role as NoV transmission vehicle (Everis, 2004), (ii) NoV survive well in the environment and probably also on raspberries (Rzezutka and Cook, 2004), (iii) NoV is characterized by a low-infectious dose accompanied with a high attack rate magnifying outbreaks (Koopmans et al., 2002), (iv) NoV is resistant to freezing.

It is reported by Cannon et al. (2006) that MNV-1, as a surrogate for human NoV, is stable in a broad pH range (pH 2–10). MNV-1 showed to be resistant to freezing on shredded onions and blanched spinach (Baert et al., 2008b). Moreover, Kurdziel et al. (2001) reported survival of poliovirus, an enteric virus which was previously used as a surrogate for NoV, on fresh raspberries stored at 4 °C for 9 days, while 1 log reduction was noticed after 8.4 days on frozen strawberries.

A heat treatment at 80 °C for 2.5 min was sufficient to inactivate MNV-1 by 6.5 log (Baert et al., 2008a). Poliovirus or FCV, used as surrogates for human NoV, caused more than 5 log reduction when a heat treatment of, respectively, 72 °C for 0.5 min (milk) or 70 °C for 1.5 min (cell culture medium) was performed (Strazynski et al., 2002; Buckow et al., 2008). This suggests that a standard pasteurization process (e.g. 88 °C for 2 min) would inactivate NoV. Yet mild pasteurization processes such as heating

at 65 and 75 °C for a short period (30–15 s) are preferred by customers to result in a minimal loss of quality of the intermediatory food product, which is in this case raspberry puree. The increased incidence of NoV outbreaks associated with raspberries raised questions of the possible role of the current minimal processing and shortened heat treatments applied in industry. MNV-1 was used as a model for human NoV in the current study to obtain quantitative data about the reductions obtained by those mild heat treatments. Similar to the validation of time/temperature treatments in food processing, no preheating up to 65 or 75 °C was considered in the experimental setup nor longer exposure to these temperatures to assume a worst case scenario in which the reduction solely originated from the actual heat treatment.

Raspberry extracts needed to be diluted 100 times before PFU could be accurately counted by the MNV-1 plaque assay. A similar effect was reported by Deboosere et al. (2004) analyzing HAV on mashed strawberries.

Less than 3 log reductions of MNV-1 were observed in this study suggesting that the risk of NoV infection remains associated with mildly pasteurized raspberry puree. A similar reduction effect (about 3 log) was reported for FCV inoculated on strawberries after treatment with 300 ppm peroxyacetic acid (Gulati et al., 2001). A disadvantage of the use of peroxyacetic acid could be discoloration of the berries which is reported at concentrations >100 ppm (Lukasik et al., 2003).

In practice, the infectivity of human NoV strains found on raspberries cannot be examined after pasteurization because of the lack of a cell culture system and the lack of a correlation between infectivity and the molecular RT-PCR detection assay (Baert et al., 2008a). Phages infecting *B. fragilis* HSP40 could be promising indicators for fecal contact of raw raspberries. Gantzer et al. (1998) found a good correlation of *B. fragilis* phages with enterovirus contamination in water. *Siphoviridae* with flexible tail such as B40-8, are able to persist in polluted water and may survive water treatments (Lasobras et al., 1997). Despite the persistence in water, B40-8 phages failed to be useful as a tool to monitor NoV inactivation during pasteurization processes. Havelaar et al. (1993) reported that the presence of the classical microbiological hygiene indicator *E. coli* is not correlated with the presence of viral contamination in the environment. Although the inactivation rates of *E. coli* and MNV-1 during mild pasteurization of raspberry puree showed a similar tendency.

Mild heat treatments applied on raspberry puree remain sensorial characteristics, but may detract from food safety and thus more attention should be given to the initial quality of raw raspberries which is obtained by good agricultural practices and good hygienic practices. In addition, alternative minimal decontamination methods retaining the raspberry nutritional value should be further evaluated for implementation in fruit processing companies.

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BRIEF REPORT

Noroviruses and sapoviruses in pigs in Belgium

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Abstract Porcine noroviruses and sapoviruses belong to the family *Caliciviridae* and are rarely reported in European countries. In this study, swine stools from a region representative of northern Europe were screened for these viruses by RT-PCR. Both porcine noroviruses and sapoviruses were detected, showing their circulation in this region. The porcine norovirus strains were genetically related to genotype 19 strains in the genogroup II of the genus *Norovirus*. The porcine sapovirus strains were genetically related to the porcine enteric calicivirus Cowden reference strain and to newly described porcine strains in the genus *Sapovirus*.

Noroviruses (NoV) and Sapoviruses (SaV) belong to the genera *Norovirus* and *Sapovirus*, respectively within the family Caliciviridae. They are small ($\sim$30 nm), icosahedral, non-enveloped viruses, with a single-stranded, positive sense, polyadenylated RNA genome. Three open reading frames (ORF) have been described in the NoV genome. Non-structural proteins are encoded by ORF1, ORF2 encodes the major capsid protein, and a second minor structural protein with a poorly determined function is encoded by ORF3. In the SaV genome, sequences coding for non-structural proteins and the major capsid protein are fused so only two ORFs are present [7]. Noroviruses and SaV are common causes of gastroenteritis in humans, and they have also been detected in several animal species: swine, cattle, mouse and lion for NoV [18]; swine and mink for SaV [8, 17]. Five genogroups (G) are currently described in both genera [6, 28], but the classification is in constant evolution. Porcine noroviruses (PoNoV) are confined to GII within the genus *Norovirus* and until recently, all porcine sapoviruses (PoSaV) to GIII within the genus *Sapovirus*. However, two other SaV genogroups have recently been proposed based on newly described PoSaV strains [25, 27]. Clinical signs reported for SaV infection in swine are benign and consist of mild, acute enteritis [9]. In contrast, PoNoVs have been detected only in adult pigs without clinical signs [26]. Classical methods of detection are based on (real-time) RT-PCR [23, 26]. Caliciviruses share properties promoting their spread, in particular their relative resistance in the environment. Therefore, the management of calicivirus disinfection can be a serious issue [5].

Human sapovirus (HuSaV) and norovirus (HuNoV) are recognised across Europe as agents of either outbreaks or sporadic cases of gastroenteritis [4, 13]. In contrast, porcine SaVs have been reported in only two European countries, namely Hungary and Italy [14, 16]. The Netherlands is the only country to have reported the presence of PoNoV in its pig population [21].

We report here the first detection of both SaV and NoV genome sequences in swine faecal samples in the same country, i.e. Belgium, a region representative of northern Europe. These sequences were genetically related to already identified PoSaV and PoNoV strains and were detected in samples from either asymptomatic or diarrhoeic pigs.

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Forty-three swine faecal samples were received from a veterinary diagnostic laboratory. Samples were collected over a period of three months (June to August 2007) at necropsy or from samples sent for laboratory diagnosis. They came from young and adult pigs from different premises in Flanders, Belgium. Clinical or necropsy signs of enteritis were recorded. Ten grams of stool was diluted tenfold in PBS complemented with 0.01% azide. Viral RNA extraction was performed using a QIAamp kit (Qiagen GmbH, Hiden, Germany), and 1 µl of each viral RNA extraction was then subjected to a one-step RT-PCR using the Quick Access kit (Promega, Madison, WI, USA). Three sets of primers were used. JV12-13 and p289–290 primer pairs were previously designed to detect HuNoV and human caliciviruses (both noro- and sapoviruses), respectively. They anneal to highly conserved regions of the genome coding for the calicivirus polymerase. A third pair, named swNo F/R, was designed by ourselves with bioinformatic tools to specifically target PoNoV sequences (Table 1).

RT-PCR amplicons were agarose gel purified with the QIAquick purification kit (Qiagen GmbH, Hiden, Germany) and cloned into pGEMt-Easy plasmid (Promega, Madison, WI, USA). Plasmid DNA was purified using a Miniprep kit (Qiagen GmbH, Hiden, Germany). Plasmid DNA sequencing of three clones for each amplicon was carried out using a BigDye terminator kit version 3.1 and resolved using an ABI 3730 automatic capillary sequencer (AppliedBiosystem, Foster City, CA, USA).

Sequences were analysed using the BioEdit Sequence Editor version 7.0 software [10]. Nucleotide similarity with the NCBI genetic database was assessed using the BLAST tool (available at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Phylogenetic inference was performed with the MEGA version 4 software package [20]. The phylogenetic trees were constructed by neighbor-joining analysis, where evolutionary distances were computed using the Maximum Composite Likelihood method. The confidence values of the internal nodes were calculated by performing bootstrap analyses with 1,000 replicates.

Five samples (PC25, PC29, PC33, PC34, PC42) gave positive amplicons by RT-PCR with the p289–290 primer pair. Using BLAST and phylogenetic analysis, sequences revealed homology with already identified PoSaV strains. Strains Sw/SaV/PC25/BE, Sw/SaV/PC33/BE and Sw/SaV/PC34/BE were genetically related to strains belonging to GIII (the PEC Cowden reference strain genogroup) within the SaV genus. Strains Sw/SaV/PC29/BE and Sw/SaV/PC42/BE were clearly genetically more divergent than other strains. The classical "GLPSG" motif of the viral RNA polymerase was predicted in each genetic sequence, and these sequences matched with strains belonging to the newly proposed GVI and VII within the SaV genus (Fig. 1). Four of the SaV sequences were detected in stools from piglets less than 8 weeks old. Two of these animals showed clinical signs of enteritis, but a bacterial aetiology (*Escherichia coli*) was also found. The two remaining animals were asymptomatic. Strain Sw/SaV/PC42/BE was detected in an older pig (16–20 weeks).

The swNo primer pair detected two PoNoV sequences. Both strains (Sw/NoV/PC23/BE and Sw/NoV/PC26/BE) were genetically related to the PoNoV genotype 19 strains (Fig. 2). These sequences were detected in stools of two fattening pigs (16–20 weeks) at necropsy. These pigs did not show any signs of enteritis. No RT-PCR amplicon was generated with the JV12-13 primers.

Whereas the circulation of human caliciviruses is widely described in European countries, the status of these countries for porcine caliciviruses has been poorly investigated. In this study, the circulation of both PoSaV and PoNoV in the same European country, namely Belgium, has been demonstrated. Genomic sequences of both PoSaV and PoNoV were detected by screening a bank of porcine stool samples with primers designed for broad detection of

Table 1 Primer pairs used in this study

Primer pair	Sequence 5′ → 3′	Polarity	Virus specificity	Nucleotide position[a]	Size of the amplicon (bp)	References
JV12	ATACCACTATGATGCAGATTA	+	NoV	4277–4297	326	[23]
JV13	TCATCATCACCATAGAAAGAG	−		4583–4603		
P290	GATTACTCCAAGTGGGACTCCAC	+	NoV, SaV	4568–4590	318	[11]
P289	TGACAATGTAATCATCACCATA	−		4865–4886		
swNo F	AGGCAGCTCTATTGGACTAG	+	PoNoV	4808–4827	355	This study
swNo R	GGTCTCATTATTGACCTCTGG	−		5142–5163		

All primers target sequences located in the 3′ region of the viral RNA-dependent RNA polymerase

bp base pair

[a] Nucleotide positions are given in comparison with the Lorsdale genome (GenBank accession number X86557)

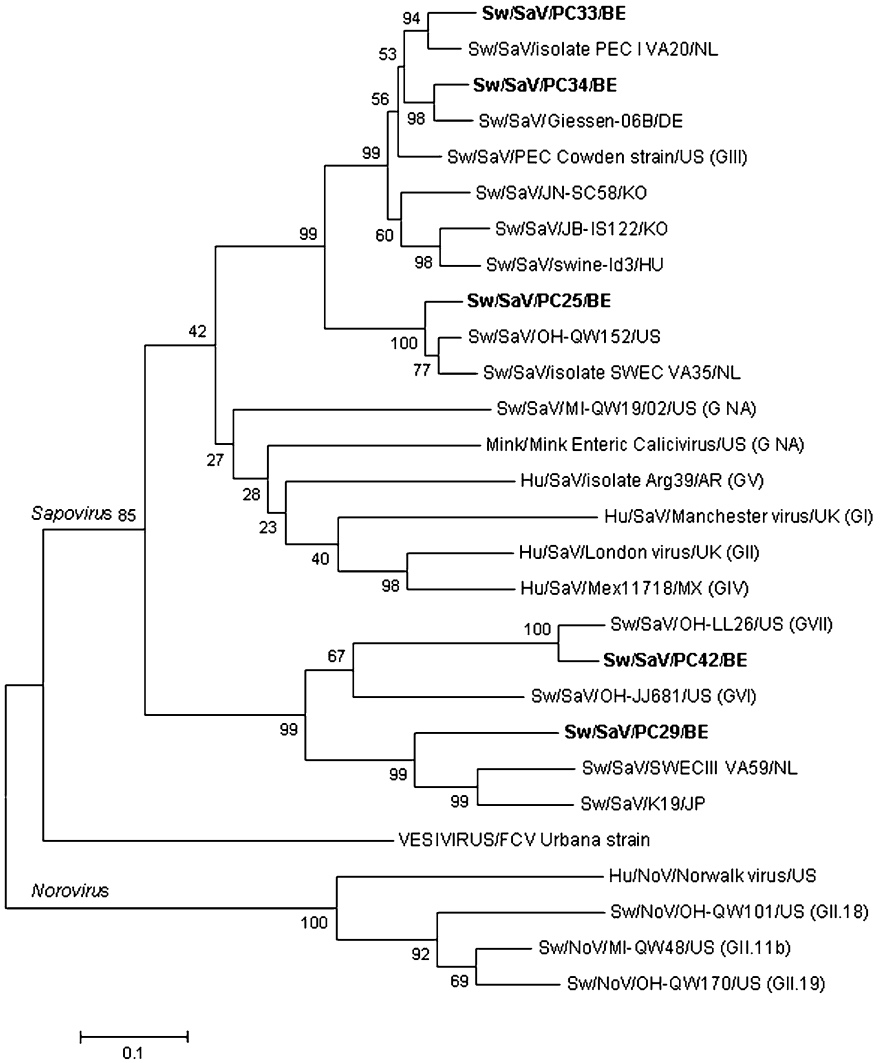

Fig. 1 Neighbor-joining phylogenetic tree based on a partial RNA-dependent RNA polymerase coding region (275 nt) of porcine sapovirus strains identified in this study (*boldface*), human and porcine sapovirus reference strains, the Norwalk virus (human norovirus) and some published porcine norovirus strains. The *scale bar* represents the phylogenetic distance expressed as expected nucleotide substitutions per site. Bootstrap values (1,000 replicates) are shown. Accession numbers in GenBank: Sw/NoV/OH-QW170/US (AY823306), Sw/NoV/MI-QW48/US (AY823303), Sw/NoV/OH-QW101/US (AY823304), Hu/NoV/Norwalk/US (M87661), VESIVIRUS/FCV Urbana strain (L40021), Sw/SaV/K19/JP (AB22304), Sw/SaV/SWECIII VA59/NL (AY615813), Sw/SaV/OH-LL26/US (AY974195), Hu/SaV/Sapporo/JP (S77903), Hu/SaV/Manchester/UK (X86560), Hu/SaV/isolate Arg39/AR (AF405715), Hu/SaV/Mex11718/MX (AY157866), Hu/SaV/London/UK (U95645), Mi/SaV/mink sapovirus/US (AF338404), Sw/SaV/MI-QW19/US (AY826424), Sw/SaV/SWEC VA35/NL (AY615808), Sw/SaV/OH-QW152/US (AY826425), Sw/SaV/swine-id3/HU (DQ383274), Sw/SaV/JB-IS122/KO (DQ389631), Sw/SaV/JN-SC58/KO (DQ389612), Sw/SaV/PEC Cowden strain/US (AF182760), Sw/SaV/Giessen-06B/DE (EU122234), Sw/SaV/isolate PECI VA20/NL (AY615804), Sw/SaV/PC25/BE (EU652844), Sw/SaV/PC29/BE (EU652845), Sw/SaV/PC33/BE (EU652846), Saw/SaV/PC34/BE (EU652847), Sw/SaV/PC42 (EU652848)

caliciviruses. No relationship to enteritis in the infected pigs could be shown. The circulation of PoSaV and PoNoV in areas with high densities of both pigs and humans raises the possibility of a zoonotic risk associated with animal caliciviruses.

Primer set p289–290 was originally designed to detect HuSaV and HuNoV sequences and targets the conserved motifs "DYSKWDST" and "YGDD" in the calicivirus RNA-dependent RNA polymerase region [11]. In this study, it amplified PoSaV sequences in swine stool samples. Surprisingly, neither p289–290 nor the JV12-13 primer pair detected PoNoV sequences. New primers (swNo F/R) designed in this study detected PoNoVs genetically related to NoV GII genotype 19 strains. The failure of detection with the p289–290 and JV12-13 primer sets most likely reflects the genetic diversity of such viruses and suggests a need for the development of more sensitive diagnostic tools.

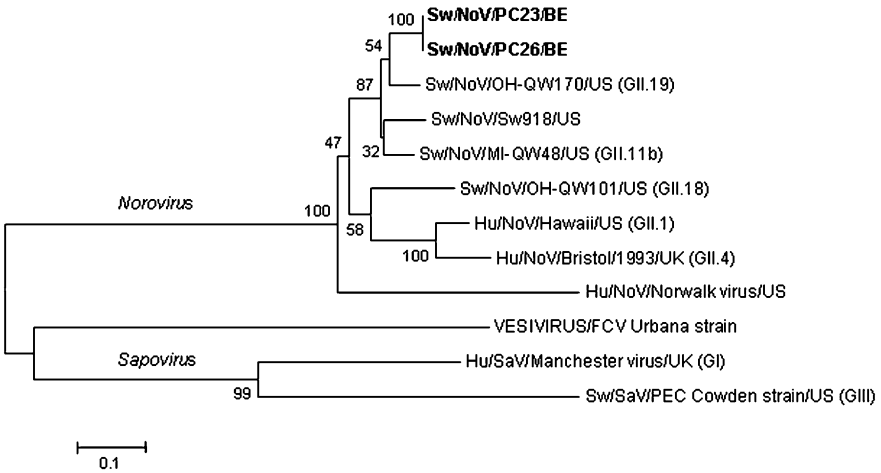

Fig. 2 Neighbor-joining phylogenetic tree based on a partial RNA-dependent RNA polymerase coding region (354 nt) of porcine norovirus strains identified in this study (*boldface*), some human and porcine norovirus reference strains, the human sapovirus prototype strain (Manchester virus) and the porcine enteric calicivirus Cowden strain. The *scale bar* represents the phylogenetic distance expressed as expected nucleotide substitutions per site. Bootstrap values (1,000 replicates) are shown. Accession numbers in Genbank: Sw/SaV/PEC Cowden strain/US (AF182760), Hu/SaV/Manchester/UK (X85560), VESIVIRUS/FCV Urbana strain (L40021), Hu/NoV/Norwalk/US (M87661), Hu/NoV/Bristol/UK (X76716), Hu/NoV/Hawaii/US (U07611), Sw/NoV/OH-QW101/US (AY823304), Sw/NoV/MI-QW48/US (AY823303), Sw/NoV/Sw918/JP (AB074893), Sw/NoV/OH-QW170/US (AY823306), Sw/NoV/PC23/BE (EU652842), Sw/NoV/PC26/BE (EU652843)

Clinically, PoSaVs were found in stool samples of both diarrhoeic and asymptomatic piglets, as already described [26]. However, a bacterial aetiology probably explains all of the clinical signs in the two diarrhoeic piglets, consistent with PoSaV being a benign enteric pathogen. Porcine NoVs were only detected in pigs without clinical signs, and the same conclusion could be stated for these viruses. To our knowledge, until now they have only been reported in adult animals. However, here, interestingly, in this study, PoNoV strains were detected in younger pigs (16–20 weeks).

Sapoviruses and NoVs are commonly reported in humans and are sometimes genetically closely related to the animal strains. Specifically, PoNoVs are genetically closely related to some HuNoVs of the GII [24], but PoSaVs are genetically more distant to HuSaV [6]. The detection of PoSaV and PoNoV suggests a potential zoonotic risk and an animal reservoir for such viruses. The circulation of PoSaV and PoNoV may be particularly prevalent in developed countries with intensive farming practices. As HuSaV and HuNoV are well distributed in the human population, it could promote the coexistence of human and animal caliciviruses in countries with high human and animal densities, e.g. in wastewater. Pigs have recently been shown to be experimentally susceptible to a human GII NoV strain [3]. Co-infection of pigs with human and porcine strains may facilitate recombination, which has been widely described in SaV and in NoV [2, 12] and proposed to be involved in the evolution of RNA viruses [1]. Swine are sometimes the host of such co-infection with other human or animal viruses and the origin of interspecies transmission [15, 22]. Furthermore, interspecies transmission involving swine has been shown for members of the *Caliciviridae* through the infection of swine with San Miguel Sea Lion virus [19]. Thus, the porcine species could potentially play a key role in any zoonotic risk associated with animal caliciviruses.

In conclusion, the circulation of both PoSaV and PoNoV was shown in Belgium, extending their European distribution. As this region is representative of the northern part of Europe, it could reflect the status of other nearby countries for both PoSaV and PoNoV. Given the uncertainties about their zoonotic risk, countries where swine and humans are kept relatively closely should develop surveillance programs where both human and animal calicivirus strains are regularly screened in gastroenteritis outbreaks, wastewater and veterinary diagnostic samples.

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Review

Animal noroviruses

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Abstract

Among enteric caliciviruses, noroviruses belong to the genus *Norovirus*, one of the four accepted genera in the family *Caliciviridae*. These single-stranded, positive-sense RNA viruses are highly variable both genetically and antigenically. Several animal enteric caliciviruses that are morphologically indistinguishable and genetically closely related to human noroviruses have been identified. The first bovine enteric noroviruses were described in Great Britain and are known as Newbury Agent 2. At least three genetic clusters of porcine noroviruses join together within genogroup II noroviruses. Human noroviruses are the most important cause of acute gastroenteritis illness in people of all ages. In the USA, they are associated with approximately 30–50% of all food-borne outbreaks. Until now, noroviruses have not been associated with gastroenteritis outbreaks in immunocompetent animals. Neither bovine nor porcine noroviruses can replicate in cell culture, although human norovirus can grow in a complex 3D culture system. However, the recently discovered murine noroviruses can replicate in cell culture and are therefore used as model viruses to study human noroviruses.

This review focusses on virus classification, virion structure, pathogenesis, epidemiology, immune response and diagnosis of animal noroviruses in comparison with human noroviruses. The classification of animal enteric caliciviruses within the *Norovirus* genus raises the question of whether transmission from an animal reservoir to humans could occur. Answering this question is important in determining the risk of cross-species infections affecting the epidemiology and evolution of these viruses and so complicating the control of human norovirus infections.

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Keywords: Norovirus; Calicivirus; Animal; Zoonosis

Introduction

Noroviruses (NoVs) belong to the family *Caliciviridae*. Caliciviruses are small non-enveloped viruses approximately 27–35 nm in diameter with a positive-sense, single-stranded RNA genome (Green et al., 2001). They have a broad host range and cause a wide spectrum of diseases and lesions in their respective hosts, including digestive tract infections (humans, pigs, cattle, dogs and mink), vesicular lesions and reproductive failure (pigs, sea lions and other marine mammal species), stomatitis, upper respiratory tract and systemic diseases (cats), and haemorrhagic disease (rabbits) (Bridger, 1990; Green et al., 2000, 2001; Guo et al., 2001; Ohlinger et al., 1993; Smith et al., 1998). Moreover, caliciviruses have also been isolated from calves with clinical respiratory signs (Smith et al., 1983).

The first discovered NoV was associated with a human outbreak of gastroenteritis in Norwalk, Ohio, which gave the name Norwalk virus (NV) to the prototype strain of NoV, in 1968 (Adler and Zickl, 1969). The virus was visualised by immune electron microscopy (IEM) in 1972 in stool samples from volunteers fed with faecal filtrates from children who were affected during the outbreak (Kapikian, 2000). At almost the same time the family *Caliciviridae* was created (Matthews, 1979). Discovery of both Norwalk virus (Kapikian et al., 1972) and rotavirus (Adams and

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Kraft, 1967; Bishop et al., 1973; Bridger and Woode, 1975) as enteric pathogens using IEM stimulated and ultimately led to the discovery of a number of enteric viruses identified as 'small round-structured viruses' (SRSV) for their general appearance under EM (Appleton and Higgins, 1975; Chiba et al., 2000; Madeley and Cosgrove, 1976).

In the early 1990s, the cloning and sequencing of the entire NV genome (strain FIIa) contributed to a new era in the study of these viruses (Jiang et al., 1993; Xi et al., 1990). The NoV virions share several characteristics, namely shedding into the faeces by patients affected with gastroenteritis, a positive-sense single-stranded RNA genome, and buoyant density of 1.33–1.41 g/cm^3 in CsCl (Kapikian et al., 1996). As molecular techniques became available in the 1990s, SRSV were divided into the Norwalk-like viruses (NLVs), now known as noroviruses, the Sapporo-like viruses (SLVs), now called sapoviruses, and the astroviruses.

Meanwhile, through the use of EM, several viruses with typical calicivirus morphology were discovered in stool samples of domestic animal species, namely calves (Woode and Bridger, 1978) and pigs (Bridger, 1980; Saif et al., 1980). Strain SW918, a prototype strain of porcine NoV, was first detected in the caecal contents of a healthy pig in Japan in 1997 (Sugieda et al., 1998). Other porcine strains were then discovered in other continents (van der Poel et al., 2000; Wang et al., 2005). Bovine NoV prototype strains identified so far are the Newbury Agent 2, first identified in the faeces of diarrhoeic calves in 1978 (Woode and Bridger, 1978), and the Jena agent, isolated in the 1980s from cattle in Germany and molecularly characterised in 1999 (Granzow and Schirrmeier, 1985; Gunther and Otto, 1987; Liu et al., 1999).

Two other enteric bovine caliciviruses have been described. These are the Newbury Agent 1 and the Nebraska (NB) strain. Newbury Agent 1 was found with Newbury Agent 2 in diarrhoeic calf samples in Great Britain and was characterised in 1984 (Bridger et al., 1984). The NB strain was detected in cattle in the USA (Smiley et al., 2002). According to Oliver et al. (2006a), the two viruses form a phylogenetically distinct clade in the *Caliciviridae* family and share 98% amino acids identity in their complete capsid protein sequence. Recently, murine NoVs (murine NoV-1, 2, 3, 4) were isolated from both immunodeficient and immunocompetent laboratory mice (Hsu et al., 2006; Karst et al., 2003) (Fig. 1). A norovirus infection was also identified in a dead lion cub in Italy (Martella et al., 2007).

Currently, no NoV has been discovered in other animal species, but some caliciviruses, related to the genus *Vesivirus*, have been found in dog faeces (Matsuura et al., 2002; Mochizuki et al., 2002; Pratelli et al., 2000). Moreover, the presence of vesivirus-specific antibodies was associated with abortion in horses (Kurth et al., 2006b) and serological evidence of vesivirus infection and also vesivirus viremia have been detected in human sera (Smith et al., 2006). Guo et al. (2001) found caliciviruses related to the *Sapovirus* genus in mink. In addition, antibodies specific to human NoVs have been detected in non-human primates (Jiang et al., 2004).

Classification

The classification of caliciviruses was first based on virus morphology. The International Committee on Taxonomy of Viruses (ICTV) proposed a new system for classification and nomenclature of the caliciviruses in 1998 and it has been further updated. The *Caliciviridae* family was divided into four genera (Green et al., 2000; Mayo, 2002): *Vesivirus*, *Lagovirus*, *Norovirus*, and *Sapovirus* and, more recently, a fifth genus, provisionally named *Nabovirus* or *Becovirus*, has been suggested (Oliver et al., 2006a) to include Newbury Agent 1 and NB virus because they show significant differences from the current four genera of the *Caliciviridae* family.

Complete sequencing of the capsid gene has allowed the classification of NoVs into five genogroups (G). Human NoV strains are found in GI, II and IV (Fankhauser et al., 2002; Green et al., 2000; Vinje and Koopmans, 2000). Bovine NoVs fall in GIII (Ando et al., 2000; Oliver et al., 2003; van der Poel et al., 2003), the murine NoVs in GV (Hsu et al., 2007; Karst et al., 2003), the porcine NoVs in GII (Sugieda and Nakajima, 2002) and the lion NoV in GIV based on partial sequencing (Martella et al., 2007).

No consensus has been reached on the classification of NoV strains within each genogroup. However a standardised method was proposed by Zheng et al. (2006) and this provides clear criteria for NoV nomenclature below the genus level using the amino acid sequences of the major capsid protein. They suggested dividing the five genogroups into 29 genetic clusters (genotypes): eight genotypes in GI (GI.1–GI.8), 17 in GII (GII.1–GII.17) – extended to 19 by Wang et al. (2007) using the same method-, two in GIII (GIII.1 and GIII.2), one in GIV and one in GV. Because recombination can affect the correct classification of NoVs (Kageyama et al., 2004), it is not recommended to use partial sequences to classify new NoV strains but rather full capsid sequencing should be performed (Zheng et al., 2006).

Based on phylogenetic analyses (Zheng et al., 2006), porcine NoVs belong to three distinct clusters in GII, which is also the most widely detected genogroup in humans. Porcine NoVs have been classified into GII.11, which is the closest to human strains, and very recently into two novel genotypes, GII.18 and GII.19 (Wang et al., 2005, 2007).

Molecular study of bovine NoVs has clarified their relationship with human NoVs, showing that they form a distinct third genogroup in the NoV genus. Historically, the first genogroup in this genus was composed solely of animal enteric caliciviruses (Oliver et al., 2003). Bovine NoV strains Jena and Newbury Agent 2 are the prototypes of genotypes GIII.1 and GIII.2, respectively (Fig. 1).

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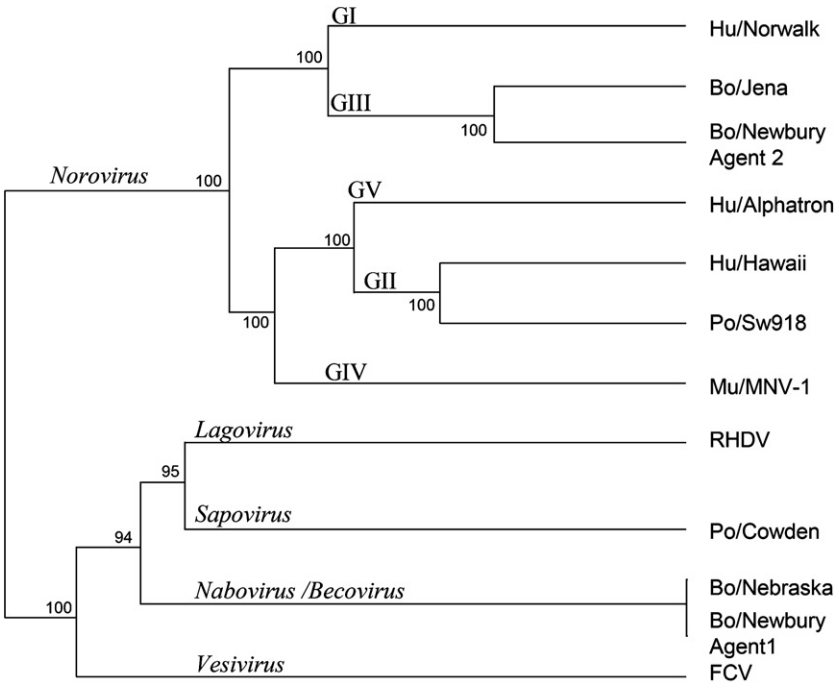

Fig. 1. Phylogenic analysis of *Caliciviridae*. Multiple alignment was performed using the ClustalW program of the partial capsid protein sequence of Alphatron norovirus (NoV) (bases 1–1668) with capsid protein sequences of representative members of the four genera of *Caliciviridae*. The numbers close to the branches indicate bootstrap values. Capsid protein sequences were derived from human NoV Alphatron (GenBank accession no. AF195847), murine NoV 1 (AY228235), porcine NoV SW918 (AB074893), human NoV Hawaii (U07611), bovine NoV Jena (AJ011099), bovine NoV Newbury Agent 2 (AF097917), bovine Newbury Agent 1 strain (DQ013304), bovine Nebraska strain (NC_004064), human NoV Norwalk (M87661), rabbit haemorrhagic disease virus (RHDV; M67473), feline calicivirus (FCV; L40021), porcine sapovirus Cowden (AF182760).

Virus and genomic organisation

Animal and human NoVs are non-enveloped, spherical particles with an indistinct surface structure and a foamy aspect outlined in EM. Studying human NoV, it was shown that the capsid is made of 180 copies of a single protein and its architecture is based on a $T = 3$ icosahedral symmetry with 90 dimers. Its surface shows 32 cup-shaped depressions and protruding arches (Prasad et al., 1999). These properties are conserved across the family *Caliciviridae* but structural variations between different members of the family *Caliciviridae* have been observed and their functional implications studied (Chen et al., 2004) (Figs. 2A and B).

The NoV genome is positive-sense, single-stranded RNA of around 7.5 kb and contains three open reading frames (ORF) (Table 1). At the 5′-end, there is a predicted genome linked viral protein (VPg) (Daughenbaugh et al., 2003). NoV possesses neither ribosomal entry site nor cap structure typical of eukaryotic mRNA but their N-terminal genome extremity is assumed to be bound to VPg, as has been described for other animal caliciviruses (Burroughs and Brown, 1978; Dunham et al., 1998; Herbert et al., 1997; Schaffer et al., 1980). In vitro, this predicted VPg interacts with components of the translation machinery (eIF3, eIF4GI, eIF4E, and S6 ribosomal protein) through unique protein–protein interactions and may play a role in initiating translation of NoV RNA (Daughenbaugh et al., 2003). There is no experimental proof of the linkage of the predicted VPg to genomic NoV RNA except for murine NoV (Daughenbaugh et al., 2006) (Fig. 3).

At the 5′-end of the genomic RNA, ORF1 encodes a polyprotein of approximately 195 kDa which is cleaved by the '3C-like' viral proteinase into at least six non-structural proteins: protein p48, which may play a role in intracellular protein trafficking (Ettayebi and Hardy, 2003); nucleoside triphosphatase (NTPase); protein p22, putatively involved in cellular membrane trafficking and replication complexes; VPg; proteinase and RNA dependent RNA polymerase (Belliot et al., 2003; Hardy, 2005). ORF2 encodes the major capsid protein (VP1) of around 60 kDa which has the following functions: self assembly and capsid formation, recognition of the receptor, host specificity, strain diversity and immunogenicity (Chen et al., 2004). A highly conserved genomic region in GI and GII NoV, including a consensus sequence of 18 nucleotides, extends from the C-terminal part of polymerase gene to the N-terminal part of the capsid coding region. This sequence could be a packaging signal for the NoV genome or a transcription initiation site (Lambden et al., 1995) and could correspond to a hot spot of recombination (Bull et al., 2005; Katayama et al., 2002).

The modular domain organisation of the VP1 subunit consists of a shell (S) and a protruding (P) domain exhibiting distinct differences. Significant structural variations are present especially in the P domain composed of P1 and P2

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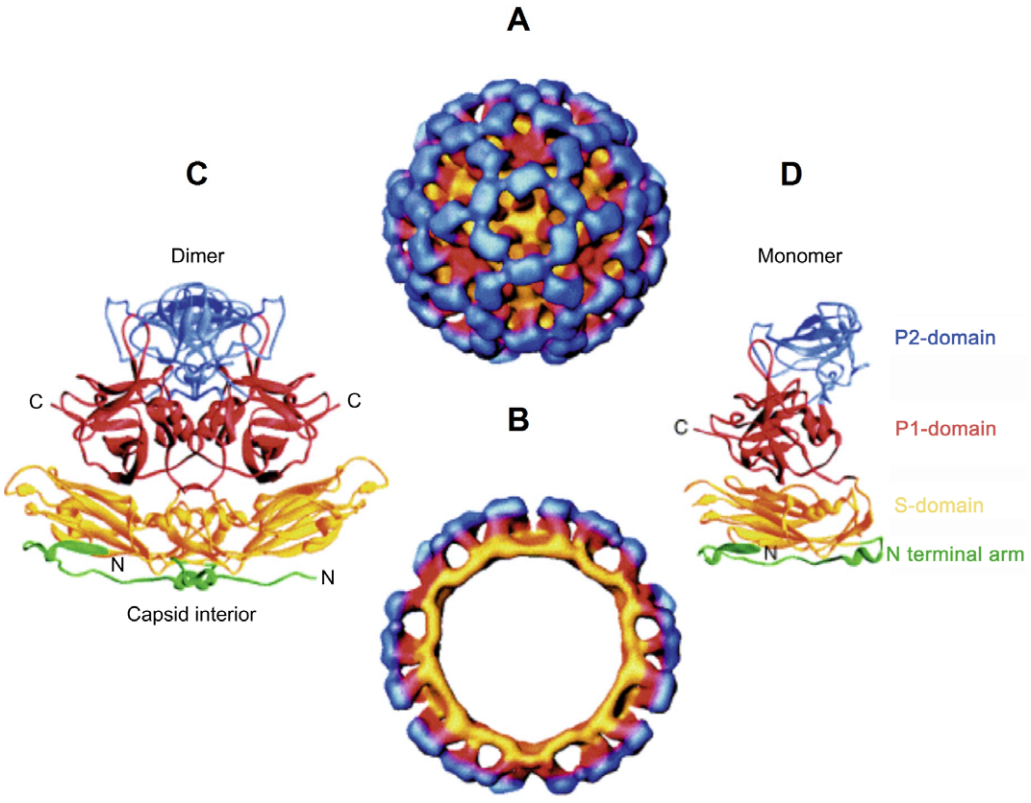

Fig. 2. Capsid structure of the Norwalk virus-like particle solved by cryo-electron microscopic at 22 Å and by X-ray crystallography at 3.4 Å. (A) Surface representation; (B) cross-section; (C) dimer of the capsid protein. Ninety dimers form the entire capsid protein; (D) Each monomeric capsid protein is organised into domains and subdomains. The N-terminal arm region (green) is facing the interior of the VLP, a shell domain (S-domain, yellow) that forms the continuous surface of the VLP and the protruding domain (P-domain) that constitutes the arch at the surface of the VLP. The P-domain is further divided into subdomains P1 (red) and P2 (blue). The latter is implicated in virus–host interactions. Adapted with permission from Hutson et al. (2004).

Table 1
Genome organisation of completely sequenced human and animal norovirus reference strains

Strain	Genogroup	GenBank access number	Genome length (nt)	Nucleotide position		
				ORF1	ORF2	ORF3
Hu/Norwalk	I	M87661	7654	5–5374	5358–6950	6950–7588
Hu/Hawaii	II	U07611	7513	5–5104	5085–6692	6692–7471
Bo/Jena	III	AJ011099	7338	22–5064	5051–6610	6600–7271
Bo/Newbury Agent 2	III	AF097917	7311	22–5076	5063–6631	6423–7271
Mu/MNV1	V	NC_08311	7382	6–5069	5056–6681	6681–7307

nt: nucleotide; ORF: open reading frame.

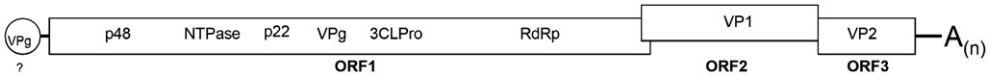

Fig. 3. Genomic organisation of noroviruses. VPg: predicted genome linked viral protein; p48: protein 48; NTPase: nucleotide triphosphatase; 3CLPro: 3C-like protease; RdRp: RNA dependent RNA polymerase; VP1: major structural protein (capsid protein); VP2: minor structural protein; ORF: open reading frame.

subunits (Chen et al., 2004) (Fig. 2C and D). P2 is the hypervariable domain of NoV capsid and its outside localisation is compatible with its function as ligand to cell receptor, found at the surface of intestinal cells (Tan et al., 2004). ORF3 which is located at the 3′-end of the genome encodes a small minor structural protein, VP2, of around 20 kDa, and is involved in expression and stability of VP1 capsid protein (Bertolotti-Ciarlet et al., 2003).

Another trait of NoVs is the accumulation and expression of subgenomic RNAs during replication in infected and cDNA transfected cells, as demonstrated for human and murine NoVs (Asanaka et al., 2005; Wobus et al.,

2004). This subgenomic expression is also used by other positive-strand RNA viruses to regulate and to allow a sufficient synthesis of structural proteins (Miller and Koev, 2000).

Virus–cell interactions

NoV infection is thought to occur in the small intestine, but no studies have identified animal noroviruses in enterocytes. NoV replication may not be restricted to enterocytes. The murine NoV revealed an unexpected tropism for the haematopoietic cell lineages, in particular macrophages and dendritic cells (Wobus et al., 2004). In the human gastrointestinal tract, intestinal dendritic cells can form transepithelial dendrites and directly acquire antigens in the lumen (Niess et al., 2005; Niess and Reinecker, 2005).

Human NoVs recognise carbohydrates linked to the human histo-blood group antigens (HBGAs), ABH and Lewis, as receptors (Hutson et al., 2002; Marionneau et al., 2002). The C-terminal region (P domain) of the capsid protein is involved in this attachment (Hutson et al., 2002; Marionneau et al., 2002; Tan and Jiang, 2005b). These carbohydrates are widely present and most mammal species express such oligosaccharides on their tissues (Marionneau et al., 2001). Receptors for animal NoVs are not yet characterised but it can be hypothesised that such molecules are involved. This hypothesis is supported by findings in other caliciviruses. For example, rabbit haemorrhagic disease virus, a lagovirus, binds to antigens of the ABH histo-blood group family (Ruvoen-Clouet et al., 2000). Interestingly, oysters, often the origin of human food-borne NoV outbreaks, express carbohydrates closely related to some HBGAs in their digestive tissues on which human NoVs could be concentrated by binding, allowing spreading when eaten (Le Guyader et al., 2006).

In vitro, human NoV-like particles were able to bind to swine gastro-intestinal washings coated on plaques (Tian et al., 2007). Furthermore, the binding of human NoVs on swine gut tissues was reported in vivo with some indication of replication (Cheetham et al., 2007). On the another hand, virus-like particles (VLP) from SW918 porcine strain (GII.11, genetically related to human NoV) did not bind to human saliva samples of the major histo-blood group types (Farkas et al., 2005). These data suggest another receptor type for this NoV, with sometimes the opportunity for human NoVs to bind to naturally expressed carbohydrates related to human HBGAs on swine gut tissues (Cheetham et al., 2006, 2007). Another explanation may be the lack of additional factors which could be essential for in vitro and in vivo NoV replication, such as those that could be provided in cell culture in a '3D-conformation' (Straub et al., 2007). Bile acids from the intestinal content are essential for the sapovirus porcine enteric calicivirus (PEC)/Cowden strain replication in cell culture by increasing cAMP concentration and down-regulation of the interferon-mediated phosphorylation of the signal transducer and activator of transcription 1 (STAT1), a key element of innate immunity (Chang et al., 2004). Moreover, recent findings imply that the proteolytic process mediated by trypsin, for example, could be necessary for human NoV replication in the host (Tan et al., 2006).

Like other members of the *Caliciviridae* family and positive-strand RNA viruses, the replication of animal NoVs could occur in association with intracellular membranes and disturb them as much as membrane associated transport (Green et al., 2002; Schwartz et al., 2004; Studdert and O'Shea, 1975). Indeed, cells transfected with a vector plasmid that provides expression of the entire NV N-terminal protein (amino acids 1-398 of the ORF1 polyprotein) showed co-localisation of this protein with cellular proteins of the Golgi apparatus (Fernandez-Vega et al., 2004). In NoV transfected and infected cells, the loss of an intact Golgi apparatus is also clearly obvious (Wobus et al., 2004). Another viral protein, VPg, can inhibit host protein synthesis (Daughenbaugh et al., 2003, 2006). In different studies, animal calicivirus RNA treatment by proteinase K abolished its infectivity suggesting an essential role of VPg (Burroughs and Brown, 1978; Dunham et al., 1998). In addition, the viral proteinase can also inhibit cellular translation by cleavage of the poly-A binding protein (Kuyumcu-Martinez et al., 2004).

Pathogenesis, clinical signs and lesions

The main transmission route is faecal–oral for both animal and human NoVs (Graham et al., 1994; Green et al., 2001; Hall et al., 1984; Hsu et al., 2005). Both epidemiological and experimental observations suggest that another natural route of infection could be the respiratory tract through aerosolised particles in vomitus (Karst et al., 2003; Sawyer et al., 1988). Caliciviruses are characterised by stability in the environment (Rzezutka and Cook, 2004) and relative resistance to inactivation (Duizer et al., 2004a). In the absence of any culture system, stability and resistance of NoVs were studied in correlation with surrogates (VLPs, Feline calicivirus). Murine NoV offers more possibilities to study these fields (Cannon et al., 2006). Low infectious doses (Graham et al., 1994) and large strain diversity (Ando et al., 2000) increase the risk of infection.

Non-hemorrhagic enteritis, mild diarrhoea, transient anorexia and xylose malabsorption were the common clinical signs reported in gnotobiotic calves infected with the bovine NoV Newbury Agent 2. Diarrhoea was more severe in 3-week-old calves than in neonates. The same clinical pattern was observed up to 2 months of age. This virus seemed to be less virulent than the other bovine enteric calicivirus Newbury Agent 1 (Bridger et al., 1984; Hall et al., 1984; Woode and Bridger, 1978). Usually, the viral shedding appeared shortly before or during the first clinical signs. Using EM, viral excretion was noted over a short period (Bridger et al., 1984), a longer faecal excretion period was seen by RT-PCR, which was more sensitive for NoV detection (Han et al., 2005; Rabenau et al., 2003).

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Histopathological lesions of calves infected with bovine NoV Newbury Agent 2 and the Jena agent consisted of villous atrophy, crypt hyperplasia and oedema in the submucosa in the proximal small intestine (Bridger et al., 1984; Gunther and Otto, 1987). Gastric and rectal mucosae were not affected (Bridger et al., 1984; Gunther and Otto, 1987; Woode and Bridger, 1978).

Porcine NoVs have been exclusively detected in faecal samples of adult swine without clinical signs (Wang et al., 2005), but in vivo studies have not been carried out. The real impact of porcine NoVs in swine diarrhoea remains to be elucidated.

Murine NoV-1 infection is asymptomatic in wildtype inbred 129 or outbred CD1 mice; on the other hand, mice lacking recombination-activating gene 2 (RAG2) and STAT1 will succumb to infection with this strain. The mice show clinical signs of encephalitis, vasculitis in cerebral vessels, pneumonia and hepatitis. In addition, the agent can be serially passaged by intracerebral inoculation, suggesting a wide NoV tropism and permissivity in immunodeficient individuals (Karst et al., 2003). Murine NoV-1 RNA was detected in spleen, mesenteric lymph nodes and jejunum from mice experimentally infected 5 weeks post-inoculation (Hsu et al., 2005). More recently, mouse lines of different immunodeficient genotypes have been infected with murine NoV-1 demonstrating systemic infections and signs of inflammation in different tissues (lung, liver, peritoneal and pleural cavities) (Ward et al., 2006). It is interesting to note that symptoms in humans are usually mild, self-limiting and of short duration (Rockx et al., 2002), except for immunocompromised, elderly or patients with underlying diseases (Goller et al., 2004; Lopman et al., 2003; Mattner et al., 2006; Okada et al., 2006). Human NoVs cause acute gastroenteritis and/or vomiting with a high secondary attack rate, especially in communities (Caul, 1996), but some human NoV case reports have documented a more severe disease with symptoms like intravascular coagulation disease or encephalitis (Brown et al., 2002; Ito et al., 2006).

In immunocompetent mice, histopathological changes are the only signs of murine NoV-1 infection (Mumphrey et al., 2007). Thus, it is assumed that disease only occurs in mice lacking components of the innate immune system (Karst et al., 2003). However, other murine NoV strains (murine NoV-2, 3 and 4) have been isolated recently in different mouse research colonies in North America (Hsu et al., 2006). These strains exhibited a different pathogenic pattern than murine NoV-1 in experimentally inoculated immunocompetent mice. While a transient infection was observed with murine NoV-1, the three novel strains showed more prolonged faecal shedding (8 weeks compared to 1 week) and signs of chronic tissue infection. This persistence could be associated with continuous replication, commonly observed with feline calicivirus (Wardley and Povey, 1977). Similar features could be suggested with other human or animal NoV strains with the outcome that asymptomatic carriers could contribute to virus dissemination and outbreaks.

Few animal NoVs cause serious clinical signs. In fact, such signs have only been noted in immunocomprised animals. In pigs, signs were only detected in asymptomatic animals. In bovines, NoVs should be viewed as benign pathogens that could facilitate or complicate gastroenteritis particularly in neonates. Only murine NoVs cause severe histopathological changes in their hosts.

Epidemiology

Epidemiological studies have repeatedly shown that NoVs are widespread and that infection is common in the human population as well as in the bovine, porcine and murine species. However, the epidemiology is not well understood and few studies have been carried out on animal NoV infections.

A serological prevalence of 22.1% was found in laboratory mice in North America, making murine NoV the most prevalent virus infecting these animals (Hsu et al., 2005). In The Netherlands, 31.6% of pooled stool specimens from veal calf farms and 4.2% of individual stool specimens from dairy cattle were positive for GIII NoV related to Newbury Agent 2 (van der Poel et al., 2003). In the UK, NoVs were detected in 11% of the cases of bovine diarrhoea tested (Milnes et al., 2007). In the US, different prevalence level of calicivirus shedding were found depending on the state: 72% in veal calves in Ohio (Smiley et al., 2003), 80% in Michigan and 25% in Wisconsin (Wise et al., 2004). In Germany, 9% of diarrhoea stool samples were positive for Jena virus whereas 99% of the serum samples collected from dairy cows were positive for the same GIII virus (Deng et al., 2003). This attests that bovine NoVs are present in cattle at a high rate in different countries.

Genogroup II NoVs were detected in pigs in Japan (Sugieda et al., 1998), The Netherlands (van der Poel et al., 2000), USA (Wang et al., 2005) and, recently, Hungary (Reuter et al., 2007). The detection rate of porcine GII NoV was low: 0.35% in Japan and 2% in The Netherlands (van der Poel et al., 2003). No circulation of porcine NoV was evidenced in Venezuela by RT-PCR screening (Martinez et al., 2006). Seroprevalence of GII NoV in swine was 97% in USA and 36% in Japan (Farkas et al., 2005).

The first putative lion NoV was detected in Pistoia zoo in Italy in a 4-week-old cub that died of severe haemorrhagic enteritis; it has not been demonstrated whether the virus was the causative agent of this enteric disease (Martella et al., 2007).

Enteric caliciviruses have been described in other domestic animal species, including cats and dogs (Herbst et al., 1987; Mochizuki et al., 1993; Schaffer et al., 1985), but until now none has been characterised as NoV.

Immune response

Initial data regarding host immune responses against NoV infection were generated by human challenge with stool filtrate or natural exposure during outbreaks, and

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have been complicated by non-immunological host factors. These may be genetic (HBGAs for human strains) and associated with susceptibility to infection (Tan and Jiang, 2005a). Early reports in humans indicated an unusual clinical immunity pattern (Blacklow et al., 1987). Human volunteer studies with NV strain established that short-term immunity, lasting about 6 months, develops against homologous viruses (Johnson et al., 1990). This short-term immunity did not necessarily extend the protection to heterologous NoV infections (Matsui and Greenberg, 2000; Wyatt et al., 1974) and could be followed by renewed susceptibility to infection 1–2 years later.

Thus, a single exposure to the virus does not necessarily confer long-term immunity (Parrino et al., 1977). Moreover, individuals with high titres of pre-challenge antibody levels to NV are not protected against reinfection and disease after a single exposure, but high antibody levels become associated with protection after repeated exposures (Johnson et al., 1990). Calves challenged with the Newbury Agent 2 showed a homologous immunity that developed 3 weeks after first inoculation (Bridger et al., 1984). To our knowledge, the duration of homologous immunity against bovine NoV has not yet been studied.

Innate immunity

Innate immunity plays an important role in the control of the murine NoV-1 infection, while B and T cell-dependent adaptive immune responses are not required for protection and STAT1 is implied (Karst et al., 2003; Wobus et al., 2004). Mice lacking both interferon (IFN)αβ and IFNγ receptors are more susceptible to NoV lethal infection than immunocompetent mice (Karst et al., 2003). In pigs inoculated with human NoV, intestinal IFNα is significantly elevated post-infection (Souza et al., 2007). In addition, self-replicating NV RNAs generated in transfected cells are sensitive to the effects of exogenous IFNα (Chang et al., 2006). The role of innate immunity in controlling infection could explain that immunodeficient subjects can develop a more severe disease and a systemic viral spread following NoV infection.

Adaptive immunity

Essentially limited to the small intestine, NoV infection stimulates the mucosal immune response and, following human experimental inoculation, a specific serum IgA response appears to be a constant feature (Erdman et al., 1989). Salivary IgAs are not cross-reactive between genogroups and could be less cross-reactive than IgG within genogroup (Lindesmith et al., 2005).

Lindesmith et al. (2003) identified two distinct patterns of NV-specific salivary IgA increase after challenge of human volunteers. Some genetically susceptible people that did not succumb to infection after challenge showed an early increase in secretory IgA, suggesting that a memory immune response could be protective. However, it is not known if this protective immunity represents short- or long-term immunity. Immune mechanisms can differ according to the animal species, but it could be assumed that a similar immunity pattern may be observed in animal NoVs. In humans, an IgM peak occurred about 2 weeks after inoculation in association with illness, and a secondary IgM response occurred in the longer term after re-challenge. An IgM response to NoV is consequently not restricted to a primary infection (Cukor et al., 1982) but is rather a marker of recent infection (Brinker et al., 1999).

Also in humans, an IgG response is activated by infection, characterised by a fourfold increase in titres (Graham et al., 1994; Lindesmith et al., 2005), which can remain high for more than 2 years after infection (Iritani et al., 2007). In calves, the antibody response raised after experimental infection is similar to the serological response observed in humans infected with human NoV. Serum IgGs are first detected at 5 days post-infection and maximum titres are reached about 3 weeks after inoculation with a genotype 2 bovine NoV (Han et al., 2005).

A characteristic of these non-enveloped viruses is the relative simplicity of the capsid. The protruding subdomain P2 is the most antigenically variable region of the capsid because it is likely to be influenced by immune pressure (Nilsson et al., 2003). Monoclonal antibodies recognising P2 epitopes block virus–cell interactions. This supports increasing evidence that interactions between NoVs and host cells rely on structures in the P2 domain of VP1 (Lochridge et al., 2005) and that VP1 possesses antigenic determinants involved in protective immunity. Inter-genogroup broadly reactive epitopes are localised in the shell domain (Batten et al., 2006; Yoda et al., 2003). Indeed, although viruses from different genogroups are antigenically distinct, bovine NoVs share a cross-reacting epitope with a human GII.3 norovirus (Oliver et al., 2006b). This epitope is localised in the N-terminal region belonging to the inner shell domain of the capsid protein that is relatively well conserved (Yoda et al., 2003).

In both pigs and humans, infection with a human GII NoV elicits a predominant but not exclusive Th1 response (Lindesmith et al., 2005; Souza et al., 2007). Numerous experiments used NoV VLPs (self assembling of VP1) as a surrogate to study NoV immunity. These VLPs are produced by different protein production systems, in particular the baculovirus system, and are structurally, morphologically and antigenically similar to the infectious virus (Han et al., 2005; Jiang et al., 1992, 1995; Le Guyader et al., 2006). VLPs are immunogenic when they are given to mice orally, intranasally or by the parenteral route (Ball et al., 1998; Guerrero et al., 2001; Jiang et al., 1992), giving rise to a systemic and mucosal immune response. Because infection with NoVs is mainly localised in the small intestine, induction of a local immunity may be important for protection against infection and disease. The conclusion from the many human studies is that immunity against human NoVs is not determined by serum antibodies (Baron et al., 1984) and pre-existing serum antibodies do

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not seem to be associated with protective immunity (Johnson et al., 1990).

Diagnosis

Electron microscopy, immunoassays (IA) and RT-PCR are used for NoV diagnosis (Lopman et al., 2002). A major problem for diagnosis using immunological or molecular techniques is the high genetic and antigenic diversity of NoVs. This is well known and described for human NoVs (Zheng et al., 2006). Genetic (Smiley et al., 2003) and antigenic (Oliver et al., 2006b; Wang et al., 2007) diversity is described in animal NoVs but is less than in human NoVs, as is seen with the number of clusters described for each NoV genogroup. This could be explained by detection bias and the development of diagnostic assays able to recognise the expected large diversity of animal noroviruses is required. Moreover, the development of diagnostic methods has been hampered by the lack of a cell culture system for NoVs (Duizer et al., 2004b), other than murine NoVs. Recently, a complex 3D-cell culture system has allowed the growth of GI and GII human NoVs (Straub et al., 2007) and this may be the beginning of a new era in NoV diagnostic tools.

First generation tests such as EM, radio-IA, Western blot or enzyme-IA have used reagents derived from previously infected humans. New generation tests following the successful cloning of NoVs have allowed the production of new reagents (such as VLPs) and new method development (such as RT-PCR) for the diagnosis of NoV infections.

Electron microscopy

Electron microscopy has been a fundamental tool for investigators, and has led to the discovery of the first NoVs, but it is a relatively insensitive method because a high viral load is necessary ($>10^6$ particles per gram of stool) (Atmar and Estes, 2001). Moreover, highly skilled microscopists are required to detect NoVs from prepared stool samples reliably. Some variants of these methods, such as IEM (Kapikian, 2000), or solid phase IEM (Dastjerdi et al., 1999), can also be used and are based on antigen–antibody reaction, visualised by negative staining EM.

ELISA

Expression of the NoV capsid protein in a baculovirus system provides large amounts of VLPs, which are used as antigens in IA. ELISA is the most widely used IA, using hyperimmune sera generated by the immunisation of animals. These assays are highly sensitive compared to EM, but their use in diagnostic laboratories is limited by their narrow specificity (Jiang et al., 2000). In fact, they are based on the detection of NoV antigens and could be hampered by antigenic diversity. ELISAs are useful because of their rapidity and simplicity for screening large number of samples. Antibody detection is more broadly reactive than antigen detection and is more suitable to identify heterotypic NoV infection (Atmar and Estes, 2001). For bovine and porcine NoVs, antibody and antigen ELISAs have been described (Cheetham et al., 2006; Farkas et al., 2005; Han et al., 2005; Oliver et al., 2007). The bovine NoVs (GIII) are divided into two serotypes, corresponding to the two distinct genotypes represented by Jena and Newbury Agent 2 strains (Oliver et al., 2006b). Antibodies against murine NoVs can be detected by ELISA (Mumphrey et al., 2007) or by a fluorescent IA (Hsu et al., 2006).

Three common epitopes shared by NoVs have been identified, one in the same genogroup, GI (Hale et al., 2000), another between GII and GIII (Oliver et al., 2006b), and the a third between GI and GIII (Batten et al., 2006). These discoveries could lead to the development of a broadly reactive antigen detection ELISA.

Reverse transcription polymerase chain reaction (RT-PCR)

Animal NoV strains can be detected by RT-PCR with primers designed for human NoVs. The assay allows the detection of porcine (Sugieda et al., 1998) and bovine (Dastjerdi et al., 1999; Liu et al., 1999; van der Poel et al., 2000) NoVs, but it is less sensitive than assays using animal NoV-specific primers. Therefore, once the presence of NoVs in animal species is proven, specific detection methods can be set up. For example, specific primers for RT-PCR and ELISA for detection of pig (Farkas et al., 2005; Wang et al., 2005) and calf NoVs (Deng et al., 2003; van der Poel et al., 2003) have been developed.

The polymerase gene is highly conserved among NoVs and numerous primer pairs have been published in this region (Le Guyader et al., 1996b; van der Poel et al., 2003; Vinje and Koopmans, 1996; Wang et al., 2006). However, analysis of more than one region is important for the detection of recombinant strains. By DNA sequencing of amplicons, information on viral phylogeny can be obtained and recombinant viruses may be detected.

RT-PCR has been developed after full length sequencing of different human NoV genomes (Jiang et al., 1993; Lambden et al., 1993). The genetic diversity among NoVs makes it impossible to develop a universal primer pair able to detect all NoVs, but some primers have been developed to detect most of circulating strains (Jothikumar et al., 2005; Le Guyader et al., 1996a; Richards et al., 2004; Vinje and Koopmans, 1996). A constant updating of primers is thus necessary.

The sensitivity of RT-PCR may be much lower than expected because of the presence of RT-PCR inhibitors in the sample (Wilson, 1997). The use of an internal control is strongly recommended to validate negative results and a few have been described for animal NoV detection in pigs (Cheetham et al., 2006; Wang et al., 2006) and cattle (Smiley et al., 2003). RT-PCR remains the 'gold standard' for NoV diagnosis because it is the most sensitive routine method used. It is being progressively replaced by real-time

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RT-PCR, which is more sensitive and faster. Various methods, such as SYBRGreen and TaqMan, have been already set up for human NoV (Jothikumar et al., 2005; Trujillo et al., 2006). These may also be used for the detection of animal NoVs, and some have recently been published for pig (Cheetham et al., 2006) and cattle NoVs (Wolf et al., 2007). Real-time RT-PCR using a Taqman probe gives the advantage of confirmation in a single assay and the opportunity of quantification if a standard is used (Jothikumar et al., 2005). This last application is of great interest as most NoVs cannot easily be cultivated in routine cell culture for plaque assay quantification.

As there is no harmonisation to compare methods (as a reference assay), the validation parameters reported in the literature are not comparable. However, a conclusion from the published assays is that RT-PCR is more sensitive than EM and ELISA (Burton-MacLeod et al., 2004; de Bruin et al., 2006; Richards et al., 2003) and it is now the mostly used assay to identify animal and human NoV infections.

The hypothesis of zoonotic risk

The detection of NoVs in animal faeces (calves and pigs), with or without clinical signs of gastroenteritis, is frequent (Ando et al., 2000; Deng et al., 2003; van der Poel et al., 2000). Molecular analyses have shown that animal and human strains are closely related, especially porcine NoVs, which are included in the same genogroup (GII) as some human strains (Oliver et al., 2003; Sugieda et al., 1998; Wang et al., 2005). Moreover, replication of a human NoV GII was recently demonstrated in gnotobiotic pigs (Cheetham et al., 2006).

Strengthening the hypothesis that animals may act as a human NoV reservoir, a high prevalence of antibodies against human NoVs was found in pigs in Venezuela. Surprisingly, a higher level of antibody prevalence against GI than GII human NoVs was observed (Farkas et al., 2005), whereas all porcine NoV detected thus far clustered with GII NoVs. These results may be explained by infection of swine with human GI NoVs or by a putative circulation of a yet undiscovered porcine NoV.

Although an animal reservoir and zoonotic transmission could exist, genetic distances (Oliver et al., 2003) and difference between receptors (Farkas et al., 2005; Hutson et al., 2003) do not support this hypothesis. Furthermore, the lack of evidence that the same strains circulate in both the human and bovine species suggests an absence of risk to human health (Oliver et al., 2003). The recent detection of sequences close to GII.4 human NoV in swine and cattle in Canada could however modify this risk evaluation in the future (Mattison et al., 2007).

Although animal NoVs have not yet been isolated from humans, human infection with NoVs related to genogroup III bovine NoV has been suggested by the presence of antibodies against bovine GIII.2 in veterinarians in The Netherlands (Widdowson et al., 2005). The existence of cross-reactive epitopes between human and bovine NoVs (Batten et al., 2006; Oliver et al., 2006b) may explain the detection of antibodies against animal NoVs in humans. Otherwise, bovine strains are unlikely to be a risk to humans because they form a third genogroup genetically distinct from human NoVs (Han et al., 2004; Oliver et al., 2003).

To date, NoV recombinants have been exclusively identified between NoVs belonging to the same genogroup and from the same animal species within bovine (Han et al., 2004; Oliver et al., 2004), porcine (Wang et al., 2005) and human species (Jiang et al., 1999; Katayama et al., 2002; Vinje and Koopmans, 2000). Bivalve molluscs present a problem in that they are filter feeders capable of concentrating viruses present in the surrounding water. Outbreaks associated with seafood are frequent, especially in countries where their consumption is high and also because they are often eaten raw (Lees, 2000). A natural co-infection with GI and GII NoVs has already been described in humans (Chan et al., 2006). Moreover, simultaneous presence of human and animal NoVs has been detected in shellfish (Costantini et al., 2006). These observations raise concern about the risk of co-infection of humans with human and animal NoVs, resulting in possible recombination and emergence of new strains.

Some animal caliciviruses are able to cross the species barrier and potentially use humans as an alternative host (Smith et al., 1998). One serotype of the Snow Mountain sea lion virus was reported to infect humans (Smith et al., 1998) and antibodies against vesivirus were found in cattle and horses (Kurth et al., 2006a; Kurth et al., 2006b).

These data suggest that appropriate conditions could be met to favour the emergence of recombinant viruses and/or an interspecies transmission of genetically compatible noroviruses.

Conclusions

NoVs are the most common cause of outbreaks of non-bacterial gastroenteritis in humans, and are also the most common cause of viral food-borne infection. In animal species, their full impact is not known, but they have already been detected in cattle, pigs and mice in several countries. As animal NoVs are closely related to human NoVs, it can be hypothesised that similar properties are shared by both viruses. Murine NoV, the only easily cultivable NoV, is useful as an animal model to study human NoVs in vitro and in vivo. Also, GII human NoVs replicate in gnotobiotic pigs, which may provide a heterologous model to study the pathogenesis of human infection. Bovine and porcine NoVs are the best candidates to be used as a homologous animal model.

To date, few complete genomes of animal NoVs have been published to better understand relatedness with viruses causing disease in humans. Of great interest for NoV researchers is the possibility of zoonotic transmission. Animal NoVs are genetically close relatives to human strains, especially porcine NoVs, which can be grouped

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with GII NoVs which are the viruses that are most frequently associated with outbreaks. The sequence similarity of porcine and bovine strains with human strains suggests that an animal reservoir of NoV infection is plausible. Co-infection and recombination between human and animal strains might occur although they have not yet been detected. Since intra-genogroup recombinants have been characterised in both human and animal species, and human and bovine NoVs have been detected in the same oysters, their co-ingestion by the same person or animal could potentially lead to the emergence of an inter-genogroup recombinant strain.

The emergence of such recombinant has the highest likelihood to occur in countries where high densities of animal and human populations where breeding practices put humans and animals in close contact so increasing the risk for cross species transmission.

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Detection and quantification of human and bovine noroviruses by a TaqMan RT-PCR assay with a control for inhibition

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ABSTRACT

Noroviruses are single-stranded RNA viruses belonging to the family *Caliciviridae*. They are a major cause of epidemic and sporadic gastroenteritis in humans and calves. Reverse transcription-polymerase chain reaction (RT-PCR) has become the "gold standard" for detection of noroviruses in faecal and environmental samples. However, false negative results due to co-concentration of RT-PCR inhibitors are a continuous concern. A TaqMan real-time RT-PCR assay making use of a foreign internal RNA control and a RNA standard was developed. Very interestingly, this method is capable of detecting human noroviruses belonging to genogroups I and II, and bovine noroviruses belonging to genogroup III. Inhibitors were removed efficiently by 1/10 dilution of the sample or addition of bovine serum albumin to the RT-PCR mix. This assay was validated with human and bovine stool samples previously tested for norovirus by conventional RT-PCR. The ability to detect norovirus in stool samples that were negative by conventional RT-PCR assay demonstrate the higher sensitivity of the TaqMan assay compared to the conventional RT-PCR assay. This real-time RT-PCR assay allows the detection of both human and bovine noroviruses, avoids false negative results and is able to quantify the level of norovirus contamination.

1. Introduction

Caliciviruses are small non-enveloped, positive-sense RNA viruses. This family is divided into four genera. Norwalk virus is the prototype strain of the Norovirus genus. This virus was first described in 1972 in association with an outbreak of gastroenteritis and vomiting involving children and staff at an elementary school in Norwalk, Ohio [1]. Other genera in the *Caliciviridae* family include Sapovirus, which also causes gastroenteritis among both children and adults, and Lagovirus and Vesivirus, neither of which are pathogenic for humans.

In recent years noroviruses have emerged as a common cause of human infectious gastroenteritis in all age groups, especially in restaurants and institutions such as nursing homes and hospitals [2–4]. They are one of the main causes of foodborne gastroenteritis [5,6]. Furthermore, several animal noroviruses genetically closely related to human noroviruses have been recently discovered [7–9]. The existence of these animal noroviruses raises important questions about potential zoonotic transmission and animal reservoirs [7]. Human and bovine norovirus diagnosis is impaired by the fact that these viruses cannot replicate in cell culture [10] although a tridimensional culture system was recently shown to be able to grow human noroviruses [11]. Reverse transcription polymerase chain reaction (RT-PCR) has been developed after full-length sequencing of different human norovirus genomes [12,13]. Many primer pairs used in RT-PCR [14], which has become the gold standard for norovirus diagnosis, have been designed. Due to the genetic diversity among noroviruses, it is very difficult to find an appropriate primer pair that is both sensitive and specific for the detection of all noroviruses. Several primer pairs are located in the most conserved region of the genome, the RNA polymerase gene [14].

Sensitivity of RT-PCR often out competes that of other techniques such as virus identification by electron microscopy and antigen-based ELISA, but false negative results can occur due to the presence of endogenous RNase in samples. Moreover noroviruses have been detected in clinical specimens (faeces and vomit) and contaminated food, water or sewage [15–18]. A number of

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commonly encountered components in such samples have been reported to be (RT-) PCR inhibitors [19,20]. Failure to adequately detect problems with either RNase contamination or RT-PCR inhibitors may result in false negative responses for samples submitted for diagnosis [21,22].

In this manuscript, we described the development of a real-time TaqMan RT-PCR assay able to detect and also quantify noroviruses circulating in the human and bovine population, an interesting fact regarding the potential zoonotic risk [7], with the advantage of pointing out samples with inhibition.

2. Materials and methods

2.1. Human and animal stool specimens

Fifty-seven human and twenty-nine bovine stool samples were tested. Human samples were selected from faeces collected over a 2-year period (2000–2002) by the Medical Microbiological and Virological Laboratory of the University hospital of Liège and from outbreaks in Belgium provided in part by the Institute for Public Health in Brussels and the Virology Laboratory of the St Luc University hospital (2006–2007). Bovine samples were taken from faeces collected by the regional animal diagnostic laboratories "ARSIA" (Association Régionale de Santé et d'Identification Animales) in Belgium over a two year period (2002–2003). All bovine and human samples had been tested previously for norovirus by conventional one-step RT-PCR (see Section 2.4) and sequenced for confirmation. The stool specimens were stored at −80 °C. All positive samples were used in this study and thirty six negative samples were randomly selected.

2.2. Viruses

Bovine rotavirus strain RF75 and human rotavirus strain WA were provided by the French food safety agency (AFSSA), Maisons-Alfort. Hepatitis A virus is from ATCC collection, ref. VR-1402. The feline calicivirus is from our collection.

2.3. Processing of stool samples and RNA extraction

Stool samples were diluted in phosphate-buffered saline (pH 7.4) to a final concentration of 10%. These suspensions were vortexed and centrifuged at 600 × *g* for 5 min. 140 μl of supernatant was used for RNA extraction using the QIAamp viral RNA Mini Kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's instructions and the rest of the supernatant was stored at 4 °C.

2.4. Conventional RT-PCR

A one-step RT-PCR kit, the Access RT-PCR System (Promega, Leiden, The Netherlands), was used with broadly reactive primer pairs which were developed for the detection of noroviruses in stool specimens from humans or bovines (Table 1). They target the polymerase or capsid protein regions of human and/or bovine noroviruses [4,23,24]. Five μl of extracted RNA was mixed with 1 μl of 50 pmol/μl primer mix. The solution was heated at 95 °C for 2 min, cooled, and 19 μl of RT-PCR reaction mixture was added. The RT-PCR reaction was performed in a final volume of 25 μl, consisting of 0.25 mM $MgSO_4$, 0.1 mM deoxynucleoside triphosphates (dNTPs), 2.5 units of avian myeloblastoma virus (AMV) RT, 2.5 units of Tfl DNA polymerase from *Thermus flavus* and 5 μl of AMV/Tfl reaction buffer (Promega, Leiden, The Netherlands). The mixture was incubated for 45 min at 48 °C for reverse transcription, followed by 2 min at 94 °C for inactivation of AMV RT and denaturation of cDNA and primers. Then, 40 amplification cycles (30 s at 94 °C, 1 min at the annealing temperature of the primer pair (Table 1), and 1 min at 68 °C) were performed followed by a final extension step at 68 °C for 7 min. The amplification products were analysed by 2% agarose gel electrophoresis and visualized with UV after ethidium bromide staining. The sizes of expected fragments are given in Table 1. All positive samples were confirmed by the sequencing of the RT-PCR products. To reduce the risk of contamination, a negative control sample was included every 18 samples. A confirmed positive human or bovine sample was included as positive control.

2.5. Real-time RT-PCR system

The real-time RT-PCR assays were carried out on the iCycler (Biorad, Nazareth, Belgium) using the iScript One-step RT-PCR kit for probes (Biorad, Nazareth, Belgium). Two μl of extracted RNA were added to 25 μl of master-mix with primers at 300 nM final concentration and probe at 150 nM final concentration.

In the first tube, a defined amount of the internal RNA control (see Section 3.1) was added with each sample of extracted RNA and primers and probe specific for the internal control (ICF, ICR and

Table 1
Primer sets and probes used in this study

Name	Sequence (5′–3′)	Annealing temperature	Size	References
Norovirus internal RNA control set up				
uSA	GTATGGCAATTGTTTCA	46 °C	491 bp	This study
lSA	ATCAGCATAAATATACGCTA			
JV12-uSA	ATACCACTATGATGCAGATTAGTATGGCAATTGTTTCA		534 bp	This study
JV13-lSA	TCATCATCACCATAGAAAGAGATCAGCATAAATATACGCTA			
Conventional RT-PCR detection of human and/or animal noroviruses				
JV12	ATACCACTATGATGCAGATTA	48 °C	327 bp	[4]
JV13	TCATCATCACCATAGAAAGAG			
CBECU-F	AGTTAYTTTTCCTTYTAYGGBA	55 °C	532 bp	[23]
CBECU-R	AGTGTCTCTGTCAGTCATCTTCAT			
CCV3	GGCTTTCCAGATTTTTCTGATTG	60 °C	407 bp	[24]
CCV4	GGCACGCTCGGAAACAAAATG			
Taqman real-time RT-PCR				
ICF	GATACACCTGAAACAAAGCATCCTAAA	48 °C	119 bp	This study
ICR	CCTTTGTCAAACTCGACTTCAATTT			
ProbeIC	TTTTCGTAAATGCACTTGCTTCAGGACCA			
SciS1	TGGGATTCAACACAACAAAGAGC		120 bp	This study
SciR1	CACCACGCTAGGAGAAAGAAGG			
ProbeSci1	CTGCGACTACCTGAGCCAAATGTGGTTC			

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ProbeIC) (Table 1). In the second tube, the TaqMan assay used the primers SciS1 and R1 and ProbeSci1 (Table 1) designed based on a norovirus strain detected in Belgium (H384) (Fig. 1). Defined dilutions of the RNA standard were tested in duplicate concomitantly with samples. The icycler RT-PCR protocol included the following parameters: reverse transcription for 13 min at 48 °C, 5 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 20 s at 48 °C and 20 s at 60 °C. To remove inhibition, bovine serum albumin (BSA) was added at a final concentration of 400 ng/μl in the RT-PCR mix or the extracted RNA was diluted 1/10.

2.6. Construction of the internal RNA control

Initially this internal control was designed to be used in conventional RT-PCR where it could be detected in the same tube with the same primer pair as noroviruses. To avoid competitive inhibition between the internal RNA control and the target viral genome, a part of the genome of *Staphylococcus aureus* was used as a foreign DNA source. Total genomic DNA of *S. aureus* was extracted and a 491 bp fragment of the *S. aureus* nuclease gene was amplified by PCR with primers uSA and lSA (Table 1). Amplified PCR products were directly cloned into a TA-cloning vector system (Promega, Leiden, The Netherlands) according to the manufacturer's instruction and the selected plasmid was then used as a template for the generation of a mutant PCR product. Primers JV12-uSA and JV13-lSA (Table 1) were used to graft the norovirus primers to the *S. aureus* fragment (Fig. 2A). Two and a half units of *Taq* DNA polymerase (Promega, Leiden, The Netherlands) was used with a 1 × PCR buffer (Promega), 0.1 mM dNTPs, 1.25 mM $MgCl_2$ and each oligonucleotide primer (final concentration of 1 μM) to make a final reaction volume of 50 μl. Thermocycling conditions were 95 °C for 5 min, followed by 40 amplification cycles, 30 s at 95 °C, 30 s at 61 °C and one minute at 72 °C, followed by a final extension step at 72 °C for 5 min. After cloning of the amplified PCR products (TA-cloning system, Promega, Leiden, The Netherlands), clone plNoSA was selected (Fig. 2) based on sequencing of the insert.

The linearised DNA clone plNoSA was used as the template for *in vitro* transcription by using the MEGAscript kit (Ambion, Huntingdon, United Kingdom) according to the instruction of the manufacturer. After overnight incubation at 37 °C, the DNA template was removed by digestion with DNase treatment with TURBO DNA-*free*™ kit (Ambion, Huntingdon, United-Kingdom). RNA was then purified with phenol:chloroform and the pellet was resuspended in nuclease-free water. After dilution, the concentrations were calculated by measuring the absorbance at 260 nm with a NanoDrop® ND1000 (NanoDrop Technologies). The RNA samples were stored at −80 °C until use.

2.7. Construction of the RNA standard for norovirus quantification

To quantify the level of norovirus contamination, the RNA standard was built using a positive human stool sample from our database, strain H384. The PCR product of 326 bp flanked by the primers JV12/JV13 [4] was directly cloned into the TA-cloning vector system (Promega, Leiden, The Netherlands) according to the instruction of the manufacturer. The clone StNoro (for standard norovirus) was confirmed to be in the correct orientation by restriction enzyme digestion of plasmids.

The linearised DNA clone StNoro was used as the template for *in vitro* transcription following exactly the same procedure as described for the internal control. RNA concentration determination and storage procedure have been previously described.

2.8. Statistical validation

Using the TaqMan assay, agreement of the viral load was established (repeatability, 53 duplicates and 5 triplicates, and reproducibility, 30 twice and 9 threefold) according to a method described by Petrie and Watson [25].

All the statistical analyses were carried out with Stata/SE [26]. The relative sensitivity and specificity were estimated with ninety-five percent confidence intervals assuming a binomial exact distribution. The limit of statistical significance of the conducted tests was defined as $P \leq 0.05$. The Kappa coefficient was calculated.

3. Results

3.1. Validation of the TaqMan RT-PCR assay for the control of inhibition

Following DNase treatment and RNA purification, the norovirus internal RNA control produced *in vitro* was detected by denaturing agarose gel electrophoresis with ethidium bromide staining (Fig. 2B). A classical PCR done to detect DNA contamination did not produce any detectable product from the undiluted RNA transcribed *in vitro*. The amount of the internal RNA control template obtained after *in vitro* transcription was 940 ng/μl.

The primers ICF and ICR (Table 1) were validated for their specificity, and failed to amplify noroviruses of the 3 genotypes, hepatitis A virus, rotavirus strains WA and RF75, and feline calicivirus. Linearity was obtained using a 10 fold dilution series of internal RNA control (from 10^5 to 10^{11} fold dilution, corresponding to 5.8×10^7 to 5.8×10^1 genome copies respectively) (Fig. 3).

```
NC001959_GI     TGGGACTCAA CACAAAATAG ACAAATTATG ACAGAATCCT TCTCCATTAT GTCGCGCCTT ACGGCCTCAC CAGAATTGGC CGAGGTTGTG GCCCAAGATT
U07611_GII      TGGGACTCAA CACAGCAGAG AGCCGTACTA GCTGCAGCCC TAGAGATCAT GGTCAAATTC TCCCCAGAGC CACACTTGGC CCAGGTAGTT GCAGAAGACC
AF097917_GIII   TGGGACAGCA CTCAACAGAG AGAGATCATG CGCCATAGCC TTGATATCAT GACCAAGCTT ACTGCAGAGC CTGAGCTCGC CCGCGTTGTT GCGGAAGACT
B309            TGGGACAGCA CCCAGCAGAG AGAGATCATG CGCCAGAGCC TTGACATCAT GACCAAGCTC ACTGCAGAAC CTGAGCTCGC CCGCGTTGTC GCGGAGGACT
H384            TGGGATTCAA CACAACAAAG AGCCGTGTTG GCAGCAGCCC TAGAAATCAT GGTTAAATTC TCCTCAGAAC CACATTTGGC TCAGGTAGTC GCAGAAGACC
SciS1           tgggattcaa cacaacaaag agc
Sci1                                                                                                    gaac cacatttggc tcaggtagtc gcag
SciR1                                                                                                                                cc

NC001959_GI     TGCTAGCACC ATCTGAGATG
U07611_GII      TTCTTTCCCC CAGTGTGATG
AF097917_GIII   TGCTTAAGCC CTCACACCTT
B309            TGCTGAAGCC CTCGCACCTT
H384            TTCTTTCTCC TAGCGTGGTG
SciS1
Sci1
SciR1           ttctttctcc tagcgtggtg
```

Fig. 1. Nucleotide sequence alignment of 3 prototype strains of noroviruses (Norwalk, Hawaii and Newbury2) with their access number in GenBank, human norovirus strain H384 and bovine norovirus strain B309 (two strains detected in Belgium) and primers SciR1 and S1, and probe Sci1. The human strain H384 was used to design the primers and probe Sci.

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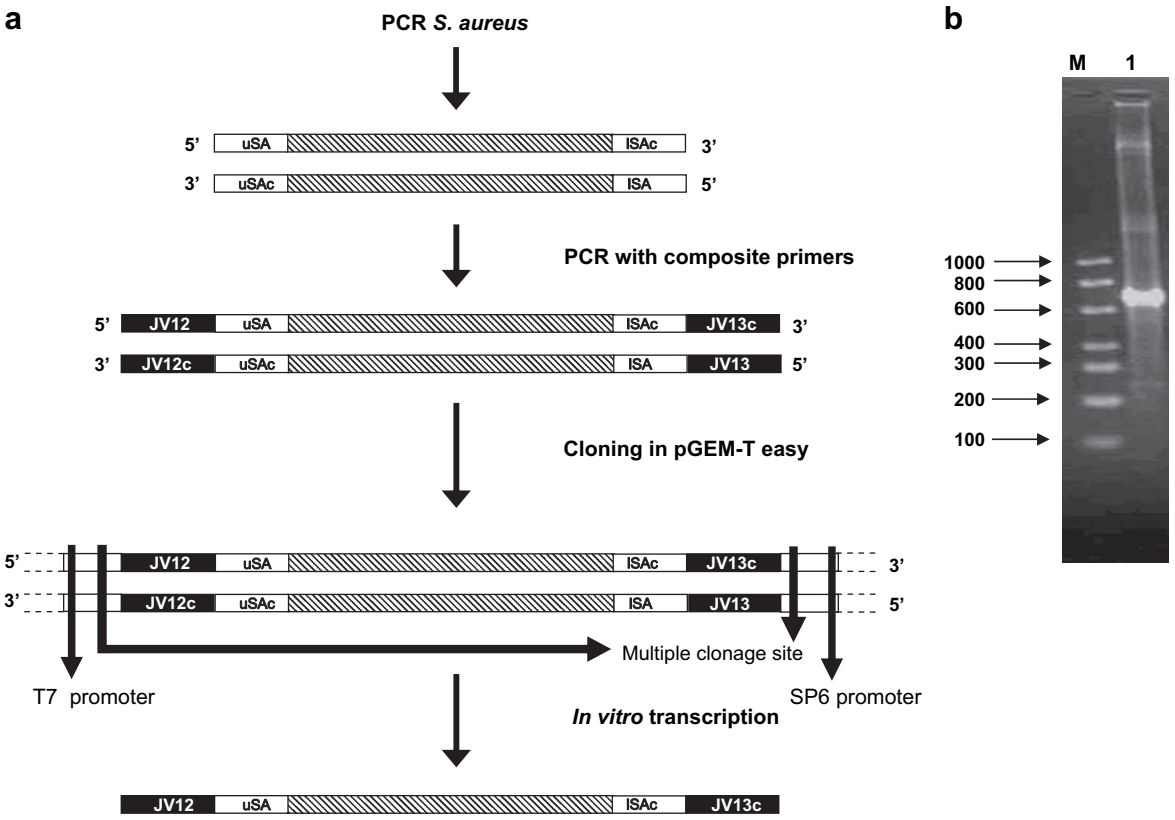

Fig. 2. Preparation of *in vitro*-transcribed RNA from *Staphylococcus aureus* containing norovirus primer sequences. (a) Schematic representation of synthesis steps showing position of oligonucleotide primer sequences. (b) Denaturing agarose gel electrophoresis of approximately 1 mg of in vitro-transcribed RNA (lane 1) with ethidium bromide staining. Lane M: RNA molecular size marker.

Serial dilution of the norovirus internal RNA control transcribed *in vitro* demonstrated that 2 μl of the 10^{11}-fold dilution contained adequate template to produce a reliable signal in real-time RT-PCR. This corresponds to a quantity of 1.9×10^{-8} ng of internal RNA control or 58 copies.

3.2. Validation of TaqMan assay for detection and quantification of noroviruses

The standard RNA was diluted in a hundred-fold dilution series ranging from undiluted to 10^{-10} and tested by classical RT-PCR and with the real-time TaqMan assay. Dilutions 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10} were kept at −80 °C and were tested in duplicate in each run in the TaqMan real-time assay. Quantities used for each dilution corresponded to 2.1×10^3, 2.1×10^5, 2.1×10^7 to 2.1×10^9 copy numbers of single stranded RNA (Fig. 4).

This real-time RT-PCR met two important objectives: it detected positive samples and quantified the norovirus viral load. Newly designed primers and probes (SciF1, SciR1 and probeSci1 (Table 1)) were validated on human and bovine stool samples which had been previously confirmed positive by conventional RT-PCR and sequencing. The analysis of dilution series showed that the TaqMan assay has the capacity to detect a quantity 10–100 times lower than the conventional RT-PCR. The specificity of the assay was tested against human WA and bovine RF75 rotaviruses, feline calicivirus and hepatitis A virus. No cross reactivity was observed. Linearity was obtained with each run of sample, using the 100-fold dilution series of RNA standard (from 10^4 to 10^{10} fold dilution) (Fig. 4).

Most of the values obtained with the TaqMan assay are within the 95% limits of the agreement (mean of differences ± 1.96 S.D. of the differences) showing satisfactory agreement. For repeatability and reproducibility, the standard error of measurement was less than 2.13 and 5.46 copies of norovirus RNA per reaction tube respectively (data not shown). The limit of detection of norovirus RNA was 52 copies per reaction tube (90% of positive replicates) using a cut-off Ct value of 40. This corresponds at least to 7.8×10^4 copies of norovirus RNA per g of stool sample.

3.3. Analysis of human and bovine stool samples

This method was able to detect the most important genogroups of human noroviruses (genogroups I and II) and genogroup III containing the bovine noroviruses. A comparison of the results obtained by conventional RT-PCR with the TaqMan real-time assay is shown in Table 2.

The use of the internal control for norovirus real-time RT-PCR diagnosis in 86 stool samples identified inhibition of RT-PCR in 15.2% of stool samples tested in this study. Two different strategies to resolve inhibition were compared: addition of BSA or 1/10 dilution of the extracted RNA. They both gave similar results.

The quantification results for human and bovine positive stool samples are given in Table 3.

Considering TaqMan assay as the gold standard (Table 2), the relative sensitivity of conventional RT-PCR was 91% (95% confidence interval (CI) 80–97). The relative specificity was 100% (95% CI: 91–100). Confirming these results, Kappa values showed a high level of agreement between the conventional RT-PCR and TaqMan real-time RT-PCR (0.88).

4. Discussion and conclusions

In this study we developed a sensitive and broadly reactive TaqMan real-time RT-PCR assay which has the unique features of detection and quantification of human genogroups I and II and

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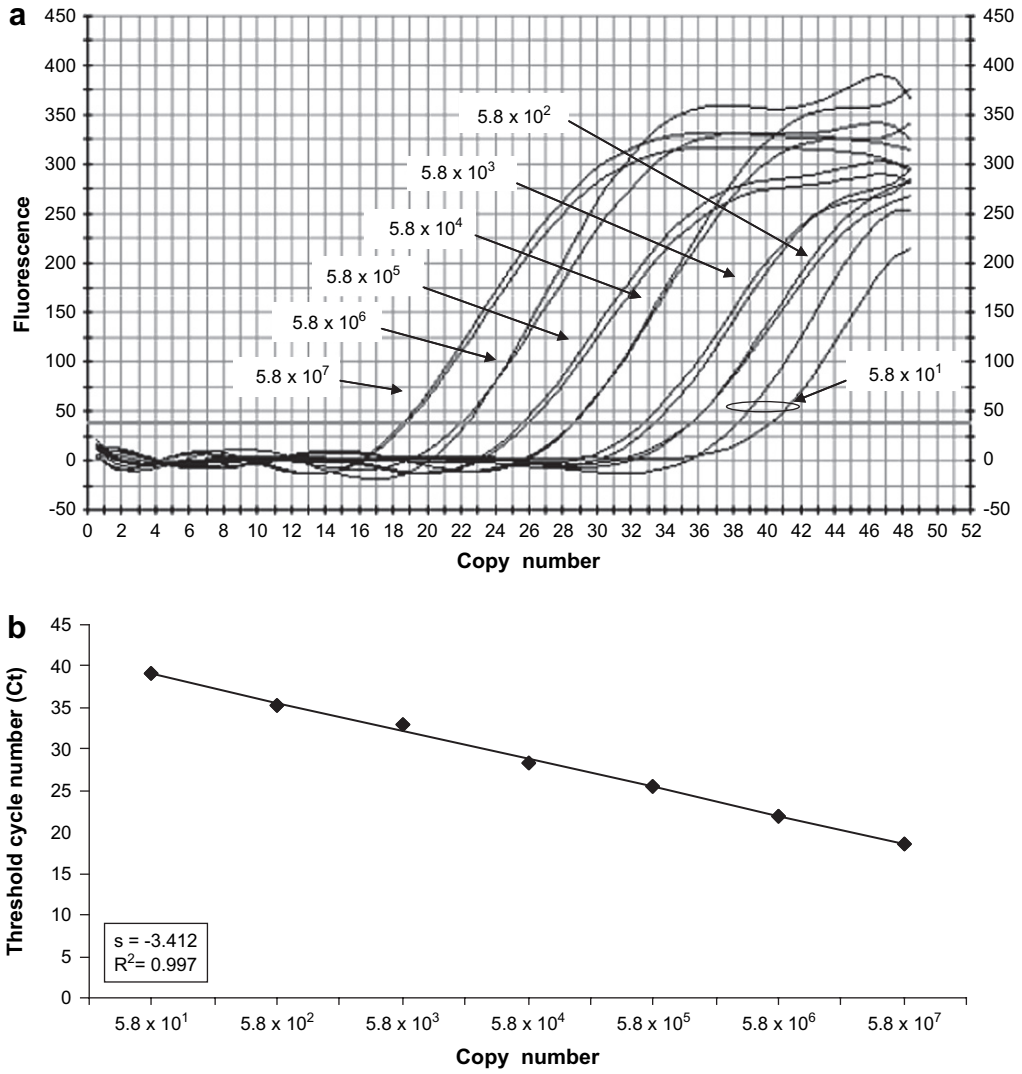

Fig. 3. (a) Detection of 10-fold serial dilution of the internal RNA control by the TaqMan assay performed in duplicate from 5.8×10^1 to 5.8×10^7. (b) Standard curve of these dilutions, each dot representing the result of duplicate amplification of each dilution. The coefficient of determination (R^2) and the slope (s) of the regression curve are indicated.

bovine genogroup III noroviruses with the same set of primers and probe. Other advantages of this assay are its ability to detect inhibitors of PCR or RT-PCR that may be present in stool [15,16] and to estimate the viral load of norovirus in the sample. The method uses a one-step, hot start, RT-PCR with thermostable DNA polymerase. The one step protocol simplifies the method and reduces the risk of contamination of RNA; moreover it is useful for routine diagnosis as there is no post-amplification processing of the product.

The genetic diversity of noroviruses makes it difficult to select a pair of primers capable of detecting all the different norovirus genogroups. The primer pair SciS1 and R1 and probe Sci1 were designed based on the sequence of norovirus strain H384 (genogroup II), in a conserved region. When tested with other human and bovine norovirus strains, viruses belonging to genogroups I, II and III have been detected. Therefore, this assay has the rare advantage of detection of norovirus strains irrespectively of their origin (human or bovine). This method is the second described that allows the detection of both human and bovine noroviruses. Compared to the method recently published by Wolf and collaborators [27], our assay has the added advantages of detection of (RT)-PCR inhibitors, the use of only one set of primers and probe for the TaqMan assay to detect human and bovine noroviruses, and the ability to quantify the contamination level.

As norovirus cannot be grown in cell culture, real-time RT-PCR using a RNA standard is the only option to quantify the viral load. In this study, the majority of positive human samples contain from 10^7 to 10^{10} copies of norovirus per gram of stool sample as estimated by the TaqMan assay (Table 3). This value is higher than those previously estimated by electron microscopy (EM) [28]. This may be due to an underestimation in EM or because the TaqMan assay is also able to detect all viral genomes, even those unpackaged in the viral capsid, whereas EM only detects full viral particles. The estimated copy number of norovirus per gram of stool sample seems lower in bovine than in human stool samples. Two hypotheses are relevant to explain that observation. First, a lower specificity of the primers and probe to genogroup III norovirus could lead to a reduced signal in the TaqMan assay. Second, this may be a specific aspect of norovirus physiopathology in bovine causing a lower level of excretion in bovine than in human. There are no available data about the quantification of bovine norovirus excretion level in bovine species. Souza and collaborators [29] showed experimental infection of gnotobiotic calves with a human norovirus and yielded an average of 7.7×10^3 GE (genome equivalent)/ml to 2×10^5 GE/ml in stool samples. Our observations with bovine noroviruses, keeping in mind that these data were obtained with another genogroup of noroviruses, are 10 times higher, from

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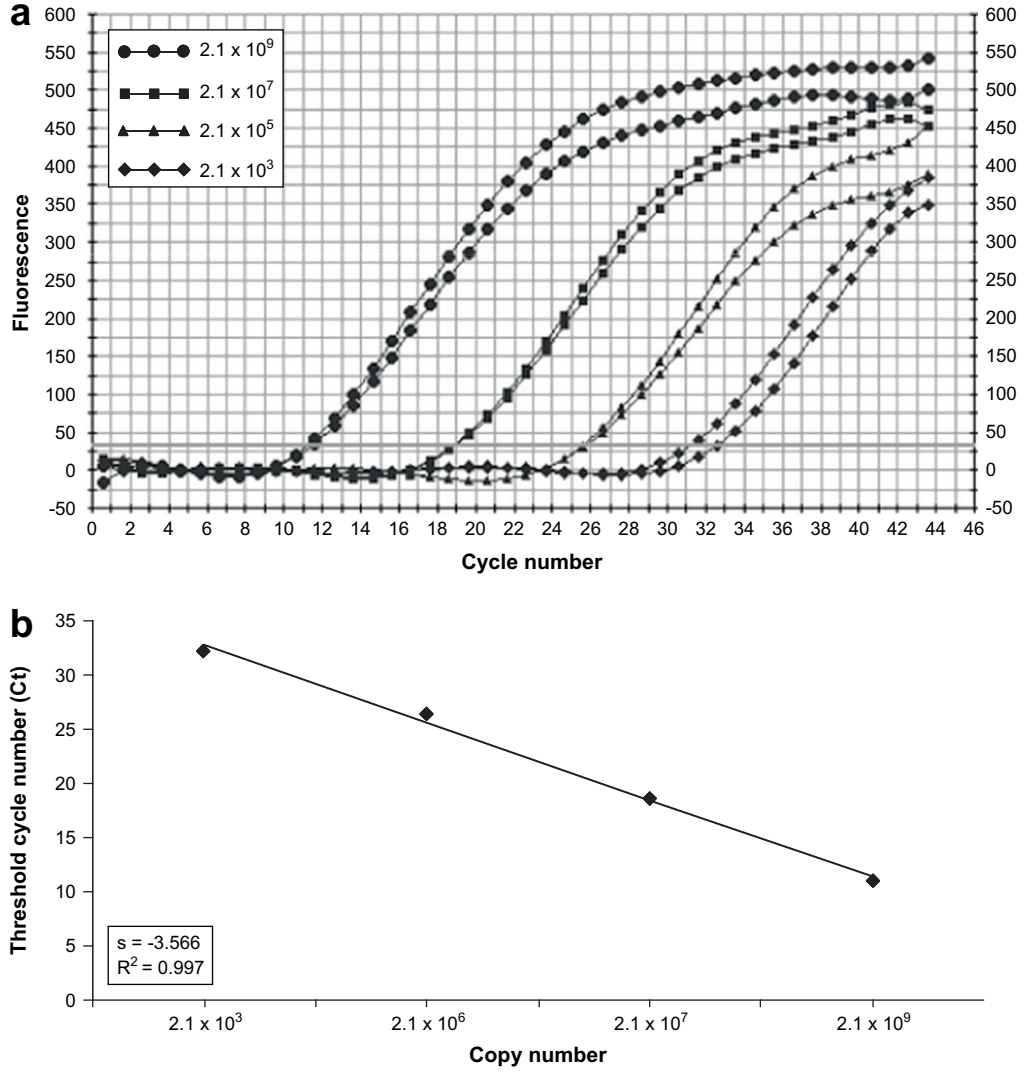

Fig. 4. TaqMan assay with the quantification RNA standard. (a) Amplification of 2.1×10^2, 2.1×10^4, 2.1×10^6, 2.1×10^8 copies of RNA standard used in parallel with each TaqMan assay. (b) Relationship between copy numbers of RNA standard, on a log10 scale, and the threshold cycle. The coefficient of determination (R^2) and the slope (s) of the regression curve are indicated.

10^4 to 10^6 genome copies/gram of stool sample. However, in both studies, at low copy numbers, quantification is less reliable, so they only represent an estimation of the excretion level.

A common problem with RT-PCR is the presence of (RT)-PCR inhibitors which may cause false negative results. Therefore, to avoid such false negative results, an internal control was set up to be used in another tube with the norovirus assay. It was synthesized *in vitro* using a foreign DNA template in order to decrease interference with norovirus amplicons [30,31]. Indeed, in another study this was very interesting because the detection was done with the same primer pair (JV12–JV13) in the same tube (data not shown). It has the advantage of representing no risk for human or animal health because it does not contain any infectious material. With this internal RNA control, inhibitors of RT-PCR can effectively be identified in samples which could be responsible for false-negative results. This improvement is crucial for early intervention and control of norovirus outbreaks.

With routine diagnostic samples tested, 15.8% of human and 13.8% of bovine samples showed presence of inhibitors. Their occurrence can vary markedly among samples and may depend on the type of sample but also on the intrinsic characteristic of sample (for example, herbivorous or omnivorous diet). When inhibition was detected in the samples used in this study, a 1/10 dilution of the extracted RNA or addition of BSA in the RT-PCR mix were tested and compared. Sample dilution is often effective as the inhibitory factors can be diluted out, however, the target nucleic acid must be in sufficient quantities in order to be detected after dilution. BSA does not have this disadvantage [32].

Out of the samples tested, the TaqMan assay showed a higher sensitivity compared to the conventional RT-PCR. In fact, five bovine samples were only detected with the TaqMan assay and were confirmed by sequencing.

In conclusion, the new serial real-time assay described in this study is an accurate, sensitive, specific and rapid method for the

Table 2
Comparison of the detection of human and bovine noroviruses by the TaqMan real-time RT-PCR and the conventional RT-PCR assay

		TaqMan assay		TOTAL
		+	−	
Conventional RT-PCR	+	50	0	50
	−	5	31	36
TOTAL		55	31	86

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Table 3
Quantification results of human and bovine Norovirus positive samples analysed using the TaqMan real-time assay

		Name	Year of detection	Quantification (genome copies/g feces)
Human	GI	H501	2003	1.51×10^6
	GII	H523	2003	2.84×10^3
		ISP55	2006	1.03×10^5
		UCL6	2006	1.04×10^5
		H425	2002	1.37×10^5
		H325	2002	2.10×10^5
		H217	2002	2.29×10^6
		H252	2002	2.91×10^6
		H7	2002	3.39×10^6
		H472	2002	8.23×10^6
		ISP58	2006	1.14×10^7
		H366	2002	1.27×10^7
		H329	2002	3.30×10^7
		ISP57	2006	3.42×10^7
		ISP477	2007	4.75×10^7
		UCL1	2007	5.61×10^7
		ISP56	2006	6.29×10^7
		H496	2003	6.46×10^7
		CrH1	2006	6.74×10^7
		H492	2003	9.84×10^7
		H556	2003	1.26×10^7
		H494	2003	1.39×10^8
		H514	2003	2.60×10^8
		H497	2003	2.62×10^8
		ISP59	2006	8.03×10^8
		H302	2002	1.60×10^9
		UCL3	2007	1.76×10^9
		UCL2	2007	2.72×10^9
		H367	2002	3.42×10^9
		CrH3	2006	3.81×10^9
		CrH2	2006	7.36×10^9
		ISP472	2007	8.00×10^9
		H361	2002	1.25×10^{10}
		ISP473	2007	1.53×10^{10}
		UCL4	2006	2.35×10^{10}
		ISP474	2007	4.15×10^{10}
		H11	2002	5.69×10^{10}
		ISP475	2007	6.54×10^{10}
		H10	2002	9.02×10^{10}
		H493	2003	3.92×10^{11}
		H384	2002	3.56×10^{15}
Bovine	GIII	B200	2003	7.06×10^4
		B214	2003	8.20×10^4
		B309	2003	1.08×10^5
		B305	2003	1.64×10^5
		B250	2003	1.90×10^5
		B52 b	2002	3.99×10^5
		B123	2002	4.95×10^5
		B307	2003	5.98×10^5
		B37	2002	1.08×10^6
		B143	2002	1.83×10^6
		B242	2003	2.88×10^6
		B128	2002	3.00×10^6
		B102	2002	4.35×10^6
		B199	2003	5.95×10^6

detection of a wide range of noroviruses belonging to genogroups I, II and III. This real-time assay offers a method to detect samples containing inhibitors and avoiding false negative results by using an internal control. This assay will be applicable for clinical diagnosis in human and animal laboratories, for detection of viruses in food or environmental samples, or as a research tool to better understand the pathogenesis of norovirus. This TaqMan real-time assay uses a single primer pair and a single probe capable of detecting and quantifying human and bovine noroviruses simultaneously. For special applications, specific probes for human and bovine noroviruses could be an interesting improvement. Nevertheless classification and study of transmission routes of noroviruses require sequencing in addition to detection. Moreover bovine noroviruses have already been detected in the food chain in a bivalve mollusc sample which was contaminated with human noroviruses [33]. This increases the risk of crossing the species barrier and the emergence of recombinant viruses and points out a real need to detect not only human but also bovine (and other animal) noroviruses.

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Methodology

A SYBR Green RT-PCR assay in single tube to detect human and bovine noroviruses and control for inhibition

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Abstract

Background: Noroviruses are single-stranded RNA viruses belonging to the family *Caliciviridae*. They are a major cause of epidemic and sporadic gastroenteritis in humans and clinical signs and lesions of gastroenteritis were reported in bovines. Due to their genetic proximity, potential zoonotic transmission or animal reservoir can be hypothesized for noroviruses. RT-PCR has become the "gold standard" for the detection of noroviruses in faecal and environmental samples. With such samples, the control for inhibition of the reaction during amplification and detection is crucial to avoid false negative results, which might otherwise not be detected. The aim of the reported method is to detect, with a SYBR Green technology, a broad range of noroviruses with a control for inhibition.

Results: A SYBR Green real-time RT-PCR assay was developed making use of a foreign internal RNA control added in the same tube. This assay is able to detect human and bovine noroviruses belonging to genogroups I, II and III and to distinguish between norovirus and internal control amplicons using melting curve analysis. A 10-fold dilution of samples appears to be the method of choice to remove inhibition. This assay was validated with human and bovine stool samples previously tested for norovirus by conventional RT-PCR.

Conclusion: This SYBR Green real-time RT-PCR assay allows the detection of the most important human and bovine noroviruses in the same assay, and avoids false negative results making use of an internal control. Melting curves allow the discrimination between the internal control and norovirus amplicons. It gives preliminary information about the species of origin. The sensitivity of the developed assay is higher than conventional RT-PCR and a 10-fold dilution of samples showed a better efficiency and reproducibility to remove RT-PCR inhibition than addition of bovine serum albumin.

Background

Norovirus is one of the four genera currently accepted into the family *Caliciviridae*. Other genera in this family include Sapovirus, which causes gastroenteritis in humans, as well as Lagovirus and Vesivirus, neither of which are pathogenic for humans. Noroviruses are small, non-enveloped viruses with a diameter of approximately 27–35 nm. They have a positive-sense, single stranded RNA genome [1]. Norwalk virus, the prototype strain of the genus norovirus, was first described in 1972 in association with an outbreak of gastroenteritis and vomiting involving children and staff at an elementary school in Norwalk, Ohio [2].

Noroviruses are now recognized as a common cause of human infectious gastroenteritis in all age groups, especially in restaurants and institutions such as nursing homes and hospitals [3-5]. They are one of the main causes of foodborne gastroenteritis [6,7]. Furthermore, several animal noroviruses genetically closely related to human noroviruses have been recently discovered [8-10]. Their existence raises important questions about animal reservoirs and potential zoonotic transmission [8]. The diagnostic of human and bovine noroviruses is impaired by the difficulties to replicate it in cell culture [11], although a tridimensional culture system was recently shown to be able to grow human noroviruses [12]. The full-length sequencing of different human norovirus genomes has allowed the development of reverse transcription polymerase chain reaction (RT-PCR) [13,14], which has become the gold standard for norovirus diagnosis [15]. Due to the genetic diversity among noroviruses, it is very difficult to find an appropriate primer pair that is both sensitive and specific for detection of all noroviruses. The most conserved region of the genome is the RNA polymerase gene and several primer pairs have been selected in that region [15], as the one used in this assay [16]. Real-time RT-PCR assays are more and more developed and has become the method of choice for the detection and the characterization of norovirus. Many different real-time RT-PCR assays for norovirus genogroups I and II had been developed [17-19] and co-detection of human and animal noroviruses was described in a multiplex assay [20] or simultaneously [21].

Noroviruses are usually detected in clinical specimens (faeces and vomit) and contaminated food, water or sewage [22-25]. Such samples commonly contain components reported to be (RT-)PCR inhibitors [26,27], leading to a high risk of false negative results or a decrease of the Ct value. A control to adequately detect problems with either RNase contamination or RT-PCR inhibitors is necessary to avoid false-negative responses for samples submitted for diagnosis [28,29]. An internal control is crucial to diagnostic (RT-)PCR assays. It is co-amplified with the target sequence and a negative result indicates a total (RT-)PCR failure. Also partial decrease of amplification capability can be estimated compared with the decrease of the internal control Ct value (internal control in the sample versus internal control alone).

The aim of this study was the development of a SYBR Green real time RT-PCR method able to detect the most important genogroups of noroviruses circulating in the human and bovine populations. This assay includes an internal RNA control and has been designed and validated for the diagnosis of noroviruses in human and bovine stool samples. Melting curve analysis allows the distinction between the internal control and norovirus amplicons and gives some indication about the species of origin. Moreover, the use of this single tube assay, cheaper than a TaqMan analysis, has the great advantages to detect (RT-)PCR inhibition that may lead to false negative results.

Results

Validation of the SYBR Green real-time RT-PCR assay – control of inhibition

The set up of the internal RNA control had been previously described [21] and the primers used in the SYBR Green real-time RT-PCR had been validated for their specificity by Vennema and collaborators [16].

Serial dilution of the norovirus internal RNA control transcribed *in vitro* demonstrated that 2 µl of the 10^9 fold dilution contained adequate template to produce a detectable product by melting curve analysis following real-time RT-PCR. This corresponds to a quantity of 1.9×10^{-6} ng of internal RNA control or 5,800 copies [21].

Amplification of the internal RNA control produced amplicons with a melting temperature 3°C lower than norovirus amplicons (Figure 1).

The exact amount of internal control to add in the mix with each RNA extraction from stool samples was determined using the detection limit of the internal control in real-time SYBR Green RT-PCR and checking the non-competitive amplification between the internal control and norovirus RNA. Different amounts of internal RNA control were added with 10-fold serial dilutions of extracted norovirus RNA. At the same time, a serial dilution of extracted norovirus RNA without internal control was tested with the SYBR Green real-time RT-PCR assay (data not shown). The quantity to add to a 25 µl mix was 3.8×10^{-5}ng of internal RNA control, corresponding to 117,500 copies. The performances of the assay were evaluated using serially diluted internal RNA control (10-fold dilutions) from 5.8×10^6 to 5.8×10^{11} copies and linearity was obtained (Figure 2).

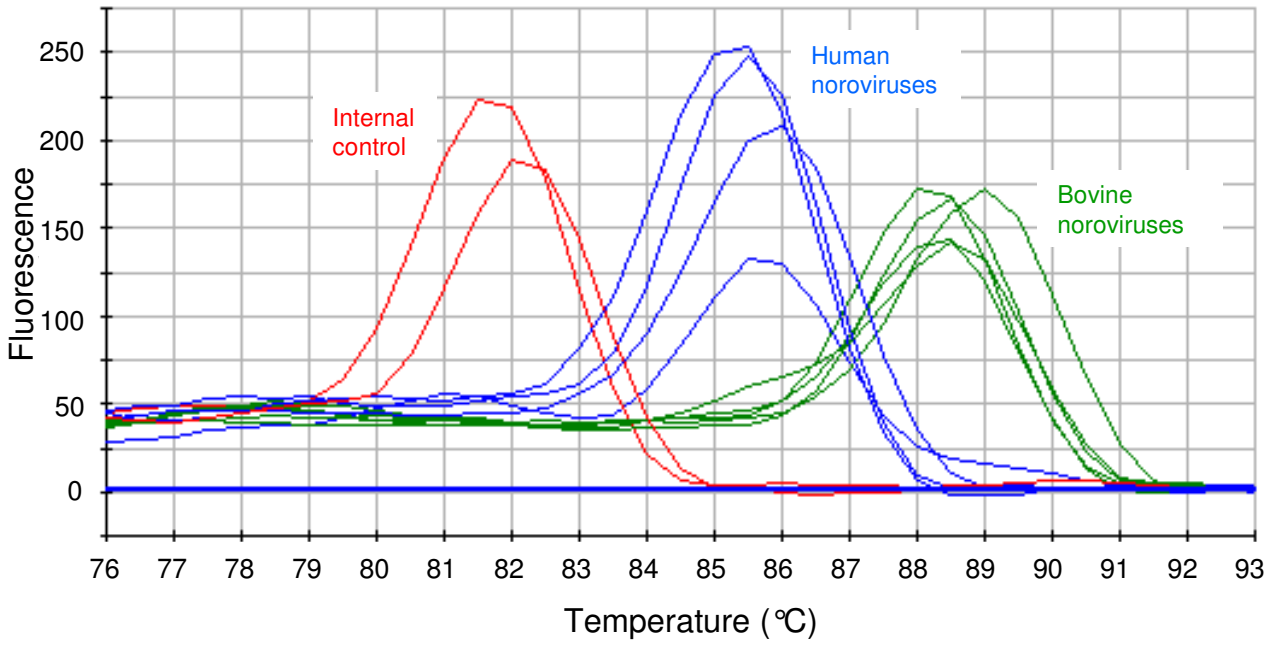

Figure 1
Distinction between norovirus amplicons and internal control amplicon allowed by the melt curve analysis. The internal control has a melting temperature around 81.5°C, human norovirus amplicons, around 85.5°C and bovine norovirus amplicons has a melting temperature around 88.5°C. Such differences in temperature are clearly visible on the curve.

Most of the values obtained with the SYBR Green assay were within the 95% limits of agreement (mean of differences +/- 1.96 S.D. of the differences) showing satisfactory agreement (data not shown). For repeatability and reproducibility, the standard errors of measurement were less than 0.291°C and 0.354°C respectively. The mean melting temperatures were significantly lower for the internal control than both human and bovine noroviruses. Moreover the mean melting temperature for human noroviruses was significantly lower than the melting temperature for bovine noroviruses (Wilcoxon rank tests, $P < 0.001$) (Figure 3).

Analysis of human and bovine stool samples

The comparison of the results obtained with the SYBR Green and the conventional RT-PCR assay is shown in table 1. In a first stage, the SYBR Green assay was performed on extracted RNA from stool samples. Three kinds of results were obtained: negative if a peak at 81.5°C was shown in the melting curve, positive if a peak around 85–88°C was shown and inhibition of reaction if there was an absence of these peaks (Figure 1). Samples containing human genogroup I or II noroviruses and bovine genogroup III noroviruses were tested and often showed different melting temperatures. The majority of bovine norovirus amplicons showed a melting temperature around 3°C higher than the human noroviruses (Figure 3).

The use of the internal control for norovirus real-time RT-PCR diagnosis in 86 stool samples identified inhibition of RT-PCR in 32.6% of stool samples tested in this study (Table 1). Two different methods were used on extracted RNA from samples showing inhibition. One is a 10-fold dilution of the extracted RNA before testing with the SYBR Green assay and the second one is the addition of bovine serum albumin (BSA) in the mix. Among the 28 samples showing inhibition, a 10-fold dilution was active in all samples compared to BSA that failed to remove inhibition from 4 samples.

Considering the SYBR Green assay as the gold standard (Table 1), the relative sensitivity of the conventional RT-PCR used in this study was 92.6% (95% confidence interval (CI) 82.1–97.9). The relative specificity was 100% (CI 91–100) for all techniques. Confirming these results, Kappa value showed a high level of agreement between conventional RT-PCR and the SYBR Green real-time RT-PCR (Kappa value: 0.90; CI 0.81–0.99).

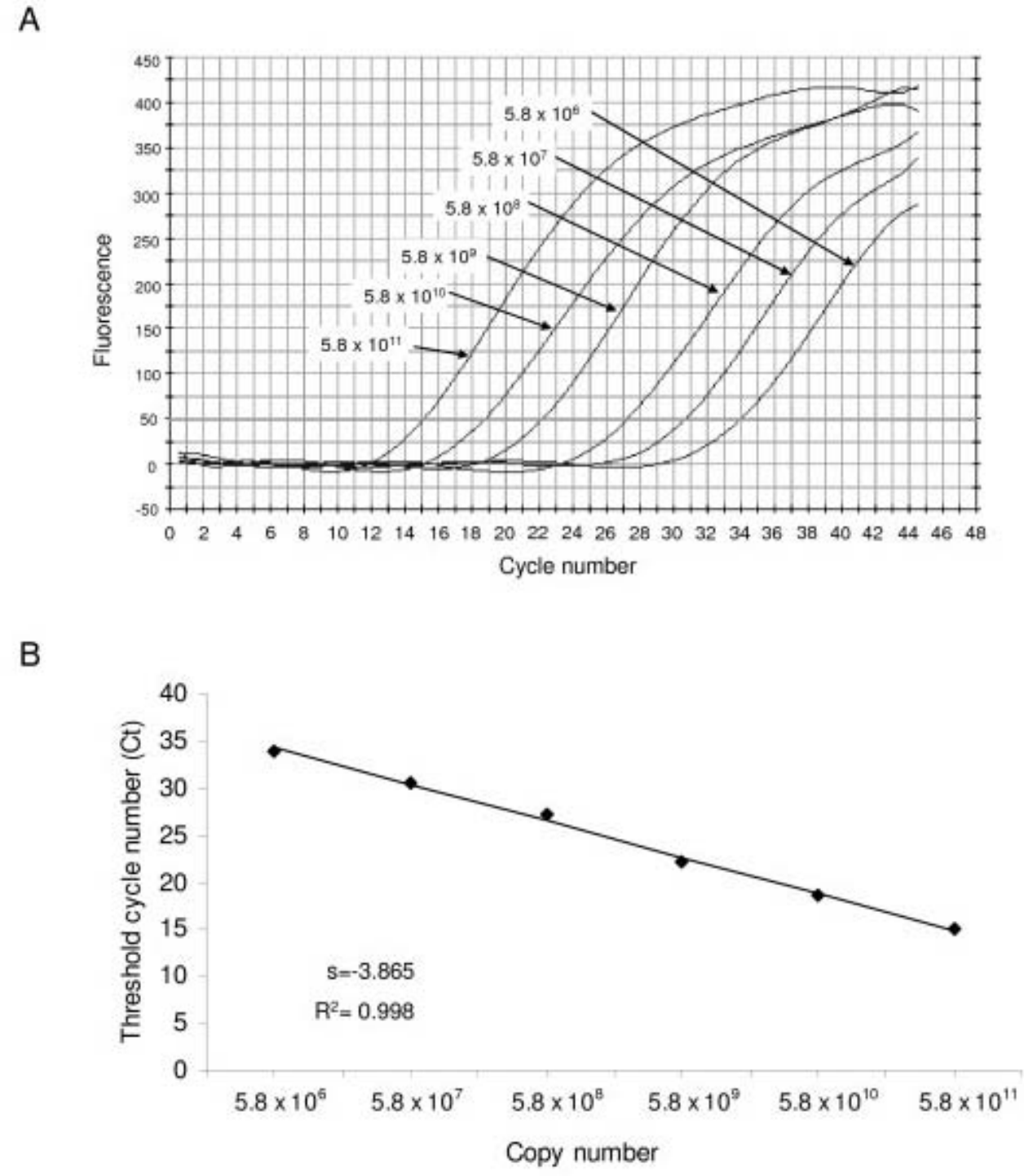

Figure 2

Linearity of the SYBR Green assay. (A) Detection of 10-fold serial dilution of a positive human sample by the SYBR Green assay performed from 5.8×10^6 to 5.8×10^{11} molecules. (B) Standard curve of these dilutions, each dot representing the result of amplification for each quantity.

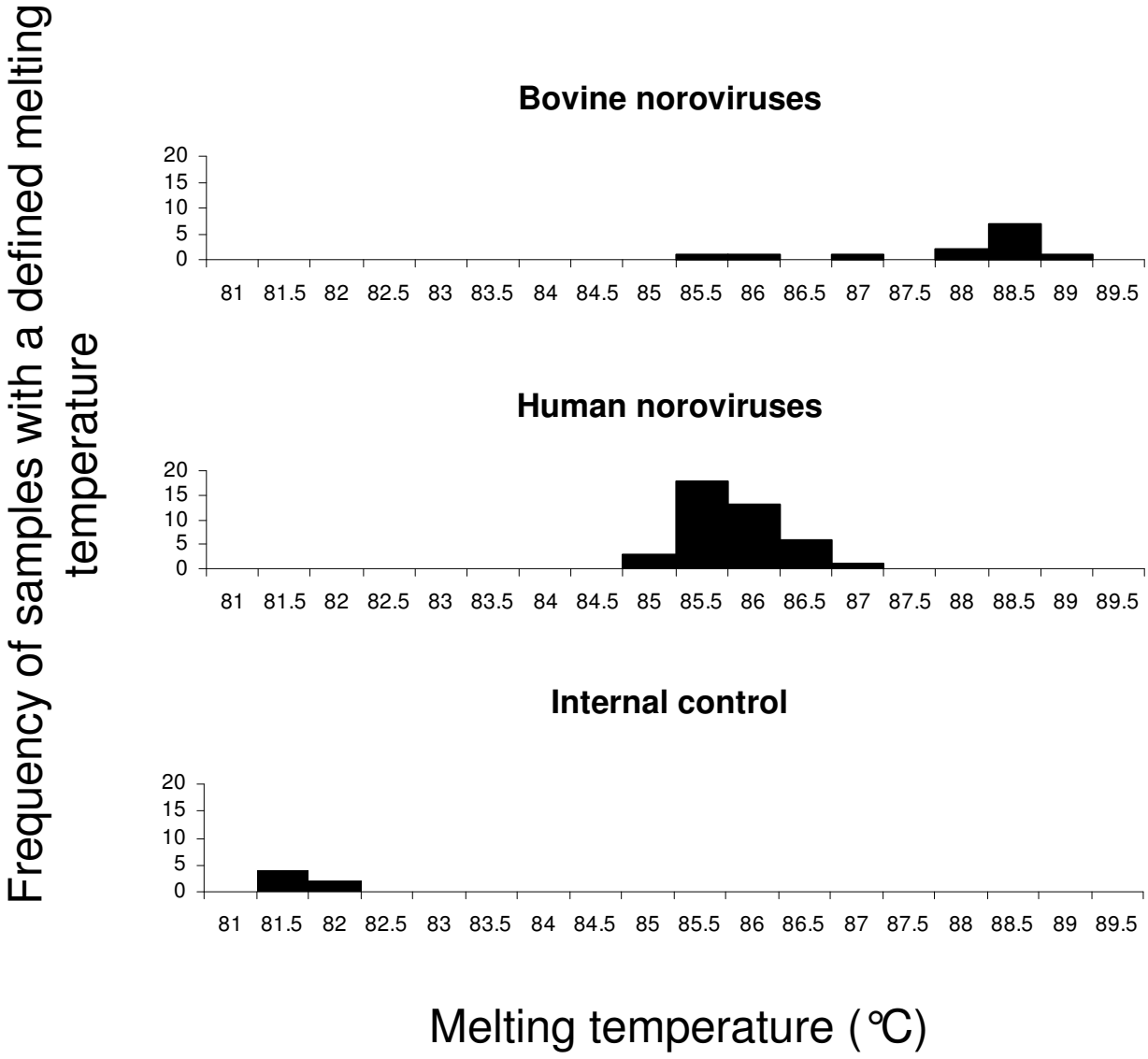

Figure 3
Frequency occurrence of amplicon melting temperature for human noroviruses, bovine noroviruses, and the internal control. The majority of bovine norovirus amplicons has a melting temperature around 88.5°C, about 3°C higher than the human ones. Though some bovine samples shown have a melting temperature similar to the human norovirus ones.

Discussion

In this study we developed a sensitive and broadly reactive real-time SYBR Green RT-PCR assay that interestingly detects human genogroups I and II and bovine genogroup III noroviruses in a single tube, including an internal control. This assay takes into account that samples may contain inhibitors of PCR or RT-PCR [22,23]. The method uses one-step, hot start RT-PCR with thermostable DNA polymerase. The one step protocol simplifies the method and reduces the risk of contamination of RNA. Moreover it is useful for routine diagnosis as there is no post-amplification processing of the product.

The genetic diversity of noroviruses makes though to select a pair of primers capable of detecting all the different norovirus genogroups. The commonly used JV12-13 primer pair has been replaced by JV12Y-13I, which contains degenerated bases to allow the detection of a larger panel of noroviruses [16]. This primer pair is able to detect noroviruses belonging to human genogroups I and II and bovine noroviruses belonging to genogroup III [30]. The variability in the melting temperature between human and bovine noroviruses can be also explained by the genetic diversity among norovirus sequences [31,32], even if the amplicon is located in the polymerase region

Table 1: Comparison of the detection of human and bovine noroviruses by the conventional RT-PCR assay and the SYBR Green assay

		SYBR Green RT-PCR						
		+		-		Inhibition		
Conventional RT-PCR	+	44	**50**	0	**0**	6	**0**	50
	-	1	**4**	13	**32**	22	**0**	36
		45	**54**	13	**32**	28	**0**	86

Normal font: SYBR Green assay on RNA directly; **bold font**: SYBR Green assay modified to remove inhibition using 10-fold dilution of extracted RNA.

highly conserved among noroviruses. Therefore, this assay has the rare advantage to detect norovirus strains irrespectively of their origin (human or bovine). There are few methods described that allow the detection of both human and bovine noroviruses. Compared to the method recently published by Wolf and collaborators [20], our assay has the advantage to detect noroviruses in the same reaction tube and to control for inhibition of the reaction. It is also to detect mixed infection (presence of human and bovine noroviruses in the same sample), by the presence of two distinct peaks.

A common problem with RT-PCR is the presence of (RT)-PCR inhibitors which may cause false negative results. Therefore, to avoid such false negative results, the internal RNA control set up previously [21] was used in this real-time SYBR Green RT-PCR assay. It was synthesized *in vitro* from a foreign DNA template, in order to decrease interference with norovirus amplicons [33,34]. It has the advantages of representing no risk for human or animal health because it does not contain any infectious material, and being stable compared to live control viruses that can evolve and change during their replication. The norovirus amplification is favored compared to the internal control because the RT-PCR of the later results in a larger product. It is an essential property for its function. It means a decrease of the Ct value of the internal control if noroviruses are present in the sample.

With this internal RNA control, inhibition can be detected without the need of additional primer pairs or an additional reaction run for this purpose and the effective identification of samples containing endogenous inhibitors of RT-PCR is allowed. This improvement is crucial for early intervention and control in norovirus outbreaks.

With routine samples used for diagnosis, 36.8% of human samples and 24.1% of bovine samples showed presence of inhibition that can vary a lot among samples and may depend on the type of sample but also on the intrinsic characteristics of the sample (for example, herbivorous or omnivorous diet). When inhibition was detected in samples, the extracted RNA was tested a second time with the SYBR Green assay, on 10-fold diluted RNA or with BSA added in the RT-PCR mix. Sample dilution is often effective as the inhibitory factors can be diluted out, however, enough quantities of target nucleic acid must be present in order to be detected after dilution [35]. The addition of BSA, that is able to scavenge a variety of inhibitory substances [36], does not have this inconvenience. Our experience in using those two techniques to remove inhibitors led to the conclusion that a 10-fold dilution is more efficient and reproducible than the addition of BSA.

Conclusion

In conclusion, the real-time assay described in this study is an accurate, sensitive, specific and quick method for the detection of a wide range of noroviruses belonging to genogroups I, II and III. At the same time it offers a method to detect samples containing inhibitors, avoiding false negative results by using an internal control. This assay will be applicable to clinical diagnosis in human and animal laboratories, detection of viruses in food or environmental samples. It is the first SYBR Green real-time assay that uses a single primer pair able to detect human and bovine noroviruses simultaneously. A 10-fold dilution of RNA appears to be the method of choice to remove inhibition.

The melting curve analysis gives presumption for the virus origin regarding the host and points out interesting samples to sequence for further studies (bovine norovirus with a melting temperature similar to the human norovirus ones). This property is of upmost importance regarding classification and study of transmission routes of noroviruses. This requires sequencing step in addition to detection. Although neither a zoonotic transmission, nor identification of an animal reservoir of norovirus have been already identified in natural condition, experimental evidence of cross infection was provided with successful inoculation of pigs with human norovirus [8,37]. Moreover bovine noroviruses have been detected in the food chain, in a bivalve mollusc sample which was contaminated with human noroviruses [38]. This increases the risk of crossing the species barrier and the probability of the emergence of recombinant viruses. In that context, a diagnostic assay that has the capacity to detect both human and bovine noroviruses is of high interest.

Methods

Human and animal stool specimens

Fifty seven human and 29 bovine stool samples were tested. Human samples were selected from faeces collected over a 2-year period (2000–2002) by the Medical Microbiological and Virological Laboratory of the Univer-

sity hospital of Liege and from outbreaks in Belgium provided in part by the Institute for Public Health in Brussels and the Virology Laboratory of the St Luc University hospital (2006–2007). Bovine samples were taken from faeces collected by the regional animal diagnostic laboratories "ARSIA" (*Association Régionale de Santé et d'Identification Animales*) in Belgium over a two year period (2002–2003). All bovine and human samples had been tested previously for norovirus by conventional one-step RT-PCR and sequenced for confirmation. The stool specimens were stored at -80°C. All positive samples were used in this study and 36 negative samples were randomly selected.

Processing of stool samples, RNA extraction and conventional RT-PCR

The all procedure was already described [21]. Briefly, stool samples were 10-fold diluted in phosphate-buffered saline and RNA was extracted using the QIAamp viral RNA Mini Kit (Qiagen, Leusden, The Netherlands). A one-step RT-PCR kit, the Access RT-PCR System (Promega, Leiden, The Netherlands), was used with broadly reactive primer pairs, developed for the detection of noroviruses in stool specimens from humans or bovines [21].

Real-time RT-PCR system

The real-time PCR assays were carried out on the iCycler (Biorad, Nazareth, Belgium) using iScript One-step RT-PCR kits for SYBR-Green assay (Biorad, Nazareth, Belgium) and used 2 μl of extracted RNA with 25 μl of master-mix with primers at 300 nM final concentration.

The primer set used was JV12Y-JV13I [16]. The quantity of 117,500 copies of the internal RNA control was added with each sample. The iCycler RT-PCR protocol included the following parameters: reverse transcription for 18 minutes at 48°C, 5 minutes at 95°C, followed by 45 cycles of 10 seconds at 95°C, 20 seconds at 48°C and 45 seconds at 60°C. Data were obtained during the elongation period. After the RT-PCR reaction, melting curve analysis was performed. To remove inhibition, BSA was added at a final concentration of 400 ng/μl in the RT-PCR mix or the extracted RNA was 10-fold diluted. A negative sample was added every 18 samples. All positive samples were confirmed by sequencing RT-PCR products.

Statistical validation

Agreement of the mean melting temperature obtained with the real-time SYBR Green assay (repeatability, 35 duplicates and 10 triplicates, and reproducibility, 33 twice and 5 threefold) was measured according to a method described by Petrie and Watson [39].

Comparison between melting temperatures obtained with the real-time SYBR Green assay in each group (human noroviruses, bovine noroviruses and internal control) was performed using Wilcoxon rank tests and assuming unequal variance and data not distributed as a normal distribution [40].

All statistical analyses were carried out with Stata/SE [41]. Relative sensitivity and specificity were estimated with 95% confidence intervals assuming a binomial exact distribution. The limit of statistical significance of the conducted tests was defined as $P \leq 0.05$ and the Kappa coefficient was calculated.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AS designed the internal control and the SYBR Green assay and did the real-time analyses. She drafted the manuscript. DZ carried out the conventional RT-PCR analyses of stool samples, and participated in analytical methods. AM was involved in the laboratory analyses and the draft of the manuscript. CS performed the statistical analysis. AS and ET conceived the study. ET is the head of the laboratory. All authors read and approved the final manuscript.

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AQ1 **REVIEW ARTICLE**

AQ2 Reported foodborne outbreaks due to noroviruses in Belgium during 2007: the link between food and patient investigations in an international context

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SUMMARY

The Belgian data for foodborne norovirus (NoV) outbreaks became available for the first time with the introduction of an extraction and detection protocol for NoV in the National Reference Laboratory for foodborne outbreaks in September 2006. In 2007, 10 NoV foodborne outbreaks were reported affecting 392 persons in Belgium. NoV became the most detected agent in foodborne outbreaks followed by *Salmonella* (eight foodborne outbreaks). The major implicated foods were sandwiches (4/10), where food handlers reported a history of gastroenteritis in two outbreaks. A food handler was implicated in the limited number of Belgian NoV outbreaks which is in accord with internationally recorded data. Forty foodborne and waterborne outbreak events due to NoV, epidemiological and/or laboratory confirmed, from 2000 to 2007 revealed that in 42·5% of the cases the food handler was responsible for the outbreak, followed by water (27·5%), bivalve shellfish (17·5%) and raspberries (10·0%).

AQ4 **Key words**: ■.

INTRODUCTION

Data on non-bacterial gastroenteritis outbreaks compiled over several years and from several countries clearly indicate the aetiological role of noroviruses (NoV). In Europe, more than 85% of the non-bacterial gastroenteritis outbreaks between 1995 and 2000 were due to NoV [1]. In The Netherlands, 76·4% of viral gastroenteritis outbreaks between 1994 and 2005 were attributed to NoV [2]. The most commonly identified transmission route was person-to-person contact followed by foodborne and waterborne spread [2]. The contribution of food or water in NoV outbreaks is generally underestimated. Under-reporting, due to a lack of appropriate detection methods for confirmation of NoV as the aetiological agent in food, often hampers identification of the actual number. In 2002, Lopman *et al.* [3] reported that only 5/10 European countries had the in-house methodology for detection of viruses in food. Currently more isolation and detection methods for NoV in foods are available but they are not easy to perform in a laboratory setting, and no official method is available [4–6].

The investigation and control of foodborne outbreaks is a multi-disciplinary task requiring information on different areas of clinical medicine,

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epidemiology, food microbiology, food safety and food control, risk communication and management. The cooperation between microbiologists providing the laboratory findings and epidemiological units is therefore of major importance. In Belgium, different authorities deal with foodborne outbreaks and as a consequence the information is dispersed and difficult to follow up. Moreover, NoV causes a self-limiting gastroenteritis; where not every ill person will visit a physician nor will all physicians request a stool sample, while not every patient will provide a sample if it is requested [7]. In Belgium NoV analysis is not reimbursed by the public health security system, which restricts the examination of stool samples for NoV. In September 2006, a NoV extraction and detection protocol was introduced in the Belgian National Reference Laboratory for foodborne outbreaks. In the last 4 months of 2006, three NoV outbreaks were detected that caused gastroenteritis in 79 persons. In 2007, 10 foodborne NoV outbreaks were identified and are discussed in the present study. Furthermore, data collected from foodborne and waterborne outbreaks between 2000 and 2007 reported by *Eurosurveillance*, *Morbidity and Mortality Weekly Reports* and internationally available peer-reviewed scientific journals are summarized. In total 40 food- and waterborne outbreak events are described of which the seasonality, the source and the role of the food handler are discussed in the present study with the objective of comparing the Belgian data with those relevant reported outbreaks.

METHODS

Collection of samples

NoV analysis was considered in food and stool samples if (i) the incubation period ranged between 12 and 48 h, (ii) acute non-bloody diarrhoea, vomiting, abdominal cramps, nausea or mild fever were present. NoV analysis was also considered in cases where an outbreak occurred with symptoms that did not correspond to the defined criteria but where shellfish was consumed or the food was prepared by food handlers before serving.

According to the symptoms of patients, the suspected food items and the quantity of food leftovers, a selection of microbiological parameters were established for investigation of the samples at the National Reference Laboratory for foodborne outbreaks in Belgium.

Laboratory investigation

Stool samples were diluted ten times in phosphate-buffered saline (PBS). The supernatant was collected after centrifuging for 15 min at 2000 ***g*** [8]. RNA extraction was performed using the RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA was eluted from the column by using 30 μl DEPC water. NoV were extracted from food items as described previously [6]. Briefly, 10 g food product was homogenized with 8 ml TRIzol® reagent (Invitrogen, Paisley, UK) allowing a contact time of 20 min (shaking) at room temperature. After centrifugation, the nucleic acid extract (supernatant) was taken and stored at -20 °C. In total, 100 μl of nucleic acid extract was purified with a RNeasy Mini kit and eluted from the column with 50 μl DEPC water. Five microlitres of the purified nucleic acid extract was used for cDNA synthesis in a RT–PCR mixture (20 μl) containing 25 U Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA, USA), 20 U RNase inhibitor (Applied Biosystems), 2·5 μM random hexamers (Applied Biosystems), 1·5 mM $MgCl_2$ (Applied Biosystems), PCR buffer II (Applied Biosystems) and 1 mM dNTPs (GE Healthcare, ■). The reverse transcription was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems) with the following cycle profile: 22 °C for 10 min, 42 °C for 15 min, 99 °C for 5 min and 5 °C for 5 min. A real-time RT–PCR detection method was applied for the detection of NoV genogroup I (GI) and II (GII) as previously described [8]. AQ5

Descriptive epidemiological information

Investigations by the Federal Agency for the Safety of the Food Chain (FASFC) are mainly focused on food-related matters whereas the communities (Flemish, French, German and Brussels) deal with people-related matters, e.g. illness. A standardized questionnaire enquiring about the suspected food, the setting where food was prepared and consumed, the processing and storage of the food and other possible contributory factors, was completed by the FASFC inspector (federal) in cooperation with the physician of the specific community and was sent to the National Reference Laboratory for foodborne outbreaks.

Genotyping NoV strains

cDNA prepared from food and stool samples was amplified using the JV12/JV13 primer couple [9]. PCR

was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems) under the following conditions: 15 min at 95 °C and 45 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min at 72 °C, followed by 10 min at 72 °C [10]. The PCR products were purified by the High Pure PCR Purification kit (Roche Diagnostics, Mannheim, Germany) and sequenced with the JV12 primer using the ABI BigDye 3.1 sequencing kit (Applied Biosystems) and an automated DNA sequencer (ABI3130XL, Applied Biosystems).

Collection of internationally reported food- and waterborne outbreaks due to NoV

Data available in *Eurosurveillance*, *Morbidity and Mortality Weekly Reports* and other international peer-reviewed journals concerning food- and waterborne outbreak events that had occurred between 2000 and 2007 were included. An outbreak event was defined as an outbreak with a common contamination source. Peer-reviewed reports were selected by the keywords: 'outbreaks' and 'viral'; 'outbreaks' and 'norovirus'; 'outbreaks' and 'Norwalk'; 'norovirus'; 'Norwalk virus' between 2000 and 2007. An attempt was undertaken to list all food- and waterborne NoV outbreaks from these sources. The authors are aware that presumably more food- and waterborne outbreaks have been reported. However, the fact that particular outbreaks were overlooked, was probably because data on these outbreaks were only published in national databases or only available in national languages and therefore did not reach a wide international public. The outbreak events reported in the present study were considered because of their detailed epidemiological information, the majority of which were well funded laboratory investigations for NoV detection. Consequently, the ongoing tendency of food- and waterborne NoV outbreaks is reflected by these reported outbreak events.

RESULTS

Ten foodborne NoV outbreaks in Belgium during 2007

In 2007, 48 food samples from 11 suspected foodborne outbreaks were analysed for NoV. In six of these outbreaks NoV was detected in the food sample analysed. In the other outbreaks no agent was detected in the food and they were therefore classified as unknown. Additionally, another four outbreaks were also classified as NoV foodborne outbreaks because of the detection of NoV in the faecal specimen and the collected epidemiological information.

In total 392 persons became ill after a NoV outbreak. Symptoms began in most cases between 12 and 24 h after food consumption with mainly vomiting, diarrhoea and slight fever reported. Hospitalization was not necessary. The majority of outbreaks occurred at work (30%), the second most important settings were at camp (20%) and in nursing homes (20%) while one outbreak each took place in a restaurant (10%), a recreation centre (10%) and at home (10%). The implicated source (food/water) of the outbreak and the number of reported cases with gastroenteritis are shown in Table 1. NoV analysis was performed in outbreaks 1, 2, 7 and 8 because specific symptoms of a NoV infection were observed. Although in six outbreaks (outbreaks 3, 4, 5, 6, 9, 10) samples (food, faecal) were examined for NoV because a food handler was involved. The role of the food handler was suspected in eight outbreaks. In one outbreak (outbreak 1) NoV was detected both in food (3/3 samples contained NoV) and in human faecal samples (1/1). Chicken with rice and soup were served to children making a daytrip to a recreation centre in January and NoV was detected in the leftovers. The unopened bags of the same production date tested negative for the presence of NoV. It is likely that an infected food handler serving the children's meal at the recreation centre was responsible for the spread of NoV through the food. Once the children returned home another 34 persons became ill with the same symptoms because of satellite outbreaks in the families (representing the second attack rate). In outbreaks 2, 4 and 7, respectively, mashed potatoes (1/1), meat stew (1/1) and a composite meal (1/2) were found positive but no stool samples were available for testing. Stool samples were not taken (outbreaks 4 and 7) or not tested for NoV presence but did test negative for bacterial pathogens (outbreak 2). Outbreak 2 occurred in an old peoples' home in March and outbreak 7 took place in a home for disabled persons in June. Outbreak 4 was located at the workplace in April. The implicated food items of outbreaks 2, 4 and 7 were handled and served by kitchen personnel before consumption and, according to the collected epidemiological information, food handlers were the suspected cause of these outbreaks.

In 4/10 (40%) outbreaks sandwiches were the most likely source of the NoV outbreak. In two of those cases (outbreaks 3, 5), history of gastroenteritis was

Table 1. *Foodborne outbreaks due to NoV reported in Belgium during 2007*

	Food/water	No. of cases involved (attack rate)	Setting	Laboratory investigation	
				Human samples	Food/water samples
1	Chicken with rice and soup	69/225 ill (30·7%)	A daytrip to a recreation centre	1/1 stool sample: GII	3/3 chicken with rice and soup: GII
2	Mashed potatoes	35/325 ill (10·8%)	Old peoples' home	Stool samples: bacteriologically negative	1/1 mashed potatoes: GII
3	Sandwiches	40/400 ill (10·0%)	At camp	5/6 stool samples: GII	No leftover sandwiches
4	Meat stew	4/132 ill (3·0%)	Institutional catering at work	No stool samples taken	1/1 meat stew: GII
5	Sandwiches	32/36 ill (88·9%)	At restaurant	No stool samples taken	1/1 sandwiches: GII
6	Chinese takeaway meal	3 ill	At home	2/2 stool samples: GII	No leftovers from the meal
7	Composite meal	70/100 ill (70%)	Institutional catering in a home for disabled persons		1/2 composite meal: GII
8	Tap water	40/105 ill (38·1%)	At camp	Stool samples: GI	
9	Sandwiches	49/200 ill (24·5%)	At work; breakfast delivered by a caterer	Stool samples: bacteriologically negative	1/1 sandwiches: GII
10	Sandwiches	16/72 ill (22·2%)	At work; sandwiches delivered by a caterer	1/1 stool samples: GI; kitchen staff member: negative	No leftover sandwiches

reported by persons preparing the food. In May a member of the restaurant staff (outbreak 5) suffered from gastroenteritis in the week before the outbreak and sandwiches (1/1), prepared by the staff including this particular food handler, tested NoV positive. Outbreak 3 took place at camp in March where a sick child assisted in the preparation of sandwiches but no leftovers from the sandwiches were available because of the late reporting (>1 week), although stool samples were NoV positive (5/6). In outbreak 9, NoV was detected in sandwiches (1/1) that were delivered by a caterer to the workplace in July. Stool samples were bacteriologically negative but not tested for the presence of NoV. Contrary to outbreak 9, NoV was detected in stool samples (1/1) collected in outbreak 10. Because of the lack of leftovers from the sandwiches implicated in outbreak 10, NoV analysis could not be performed. One outbreak (outbreak 6) occurred at home in May. After eating a Chinese takeaway meal, three persons showed symptoms of gastroenteritis. Stool samples indicated the presence of NoV (3/3). Outbreak 8 was the only suspected waterborne outbreak at a camping site in July. Epidemiological information indicated tap water as the most likely source of the outbreak. However, due to the lack of an appropriate concentration/extraction method of NoV for water, negative results were obtained.

NoV genogroup I (GI) was associated with two outbreaks (outbreaks 8 and 10) while the majority of the outbreaks were due to NoV GII (8/10). NoV detected in three stool samples originating from outbreak 3 were sequenced and revealed NoV/GII.4/Terneuzen70/2006 with 99% similarity. One stool sample was sequenced in outbreak 6 and showed a similarity of 97% with No/GII.2/Kuenzelsau/3870/05. No other faecal material was available for sequencing. It was not possible to obtain sequence data from food samples.

Internationally reported data of food- and waterborne NoV outbreaks from 2000 until 2007

International food- and waterborne outbreak events that occurred between 2000 and 2007 were compared with the Belgian results obtained in 2007 [11–48]. In total 40 events of food- and waterborne outbreaks were included of which 29 outbreaks (72·5%) took place in Europe and 11 outside Europe (27·5%). Four

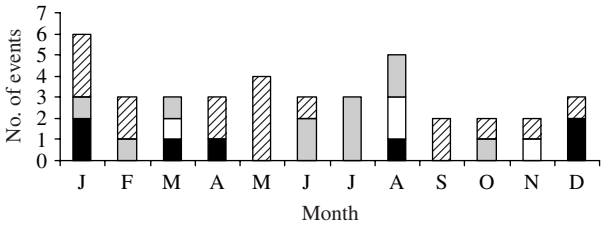

Fig. 1. The number of international food- and waterborne outbreak events according to the source and month. Histograms show outbreaks due to raspberries (□), bivalve shellfish (■), water (▣) or handling by a food handler (▨).

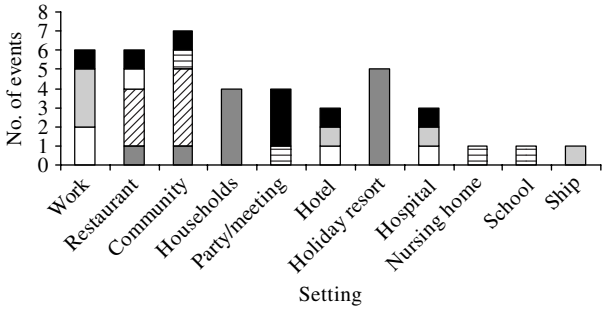

Fig. 2. The number of international food- and waterborne outbreak events according to the place and suspected food item. The implicated food items are classified in categories: raspberries (▤), sandwiches (□), salads (▣), water (▩), shellfish (▨), other food items (■). AQ11

reported events were attributed to contaminated raspberries (10·0%). One of these four events included four outbreaks that were linked in Sweden by a common origin of contaminated raspberries [47]. In another event, six outbreaks were clustered in Denmark [45]. Bivalve shellfish was defined as the source in 7/40 listed outbreak events (17·5%). Although three out of those seven events included several clusters of outbreaks whereby each cluster within an event had a common contamination source. Eleven reports were water associated (27·5%). Five waterborne outbreaks indicated environmental water as the origin of the outbreak. Six other waterborne outbreaks took place through consumption of contaminated drinking water.

For 17 outbreak events (42·5%), the role of a food handler was implicated as the origin of the outbreak. One outbreak event in the United States indicated three clustered outbreaks due to a caterer, where one of the food handlers with history of gastroenteritis was involved in three separate catered meals at work [21].

Figure 1 depicts the number of outbreaks in the year and shows that outbreaks took place throughout the year. Outbreaks were additionally categorized according to the place and the implicated food item (Fig. 2). Sandwiches and salads were the major implicated food items at work. Shellfish was responsible for the majority of outbreaks in restaurants and households. Outbreaks that occurred in the community and in holiday resorts were caused by water. Regarding parties and meetings, food prepared by caterers was the most important source of NoV outbreaks.

Table 2 shows the implicated food/water item, attack rate and concise laboratory results of 40 international outbreak events including 50 detailed outbreaks. From these outbreaks 16 did not determine sequences, of which 15 did not even report the specific genogroup. Food handler-associated outbreaks where the NoV genogroup was known, revealed the presence of GI in four outbreaks and GII in seven outbreaks. None of the outbreaks revealed both genogroups. Conversely, both GI and GII were frequently detected in shellfish, water and raspberry outbreak events.

Attack rates mentioned in Table 2 varied between 8·8% and 100% for food handler-associated outbreaks. Attack rates of 58–74·1%, 31·5–76·6% and 27·8–100% were involved in shellfish, water and raspberry outbreaks, respectively.

DISCUSSION

Ten out of the 75 reported foodborne outbreaks in Belgium during 2007 were due to NoV and these exceeded the number of *Salmonella* outbreaks (eight reports). Reporting of foodborne outbreaks to the EFSA is mandatory for European member states. In 2006, 53·9% of the reported foodborne outbreaks by 25 European countries were due to *Salmonella*. *Campylobacter* (6·9%) was the second most commonly reported causative agent followed by caliciviruses (6·2%) [49]. The introduction of clinical profiles specific for NoV in foodborne outbreaks of unknown aetiology reported to the Centers for Disease Control and Prevention (CDC) between 1982 and 1997, showed an increase from 1% to 38% of NoV outbreaks, even exceeding outbreaks with *Salmonella* as the causative agent [50]. Consequently, these numbers indicate that NoV is an important causative agent of foodborne outbreaks, not only in Belgium, but also in Europe and the United States.

Table 2. *Internationally reported foodborne and waterborne outbreaks from 2000 to 2007*

Food/water	Type of evidence	Attack rate	Laboratory investigation		Ref.
			Human samples	Food/water samples	
Food handler					
Salami + ham	Conf.	40 people ill	4/4 stool: GII.b	Salami + ham: GII.b	[11]
Spare ribs	Pres.	100 %		Spare ribs: GII.b	
Ham		55·0 %		Ham: GII.4	
Salad		48·9 %	3 stool: GI		[12]
Food		72·1 %	7/8 stool GI		[13]
Food		8·8 %	19/19 stool: NoV		[14]
Food		58·3 %	12/13 stool: GII.4		[15]
Sandwiches/salads		40 people ill	12/14 stool: GI		[16]
Wedding cake		39·1 %	Stool: NoV		[17]
Lunchboxes		91 staff members ill from 8 companies	21/23 stool: GI.12		[18]
Sandwiches	Cohort study	66·7 %	14/18 stool: NoV		[19]
Salads		44·2 %	32/59 stool: GII		[20]
Lettuce		3 cases: OB 2: 57·9 %			[21]
Submarine sandwich		OB 3: 50·0 %			
Submarine sandwich	CC	OB 1: 79·3 %			[21]
Sandwiches		27·0 %	15/16 stool GII		[22]
Prepared salad		56·0 %	5/6 stool: NoV		[23]
Rice salad with cocktail sauce		38 people ill	2/4 stool: NoV		[24]
Mixed salad		36 ill cases defined	3/9 stool: 2 GII.4variant 2 + 1 GII.4variant 3		[25]
Shellfish					
Clams	Pres.	5 ill		59 pooled clams: GII	[26]
Oysters		68·2 %	11/11 stool: NoV: 2 GI types and 3 GII types and 1 mixed infection		[27]
Oysters (Italy)		202 people ill (Italy)	7/12: stool GI.4, GII.4,GII.b		[28]
Oysters (France)	Conf.	58 %	22/41 stool: GI.4,GI.6, GII.4, GII.8	3/3 shellfish: GI.4, GII.4, GII.8	
Oysters		14 cases (small clusters), only 1 outbreak with 4 consumers investigated	2/4 stool GI.1	5/6 shellfish: GI.1	[29]
Frozen half-shelled oysters		14 cases, 305 people ill; attack rate: > 82 %	4/5 stool: GII	6/11 oysters: GII	[30]
Mussels		74·1 %	24/24 stool: GI, GII	6/11 mussels: GI, GII	[31]
Oysters		53 people ill	26/53 stool: GI.2	Oysters: GI.2	[32]
Water					
Flood water	Pres.	76·6 %	4/7 stool: NoV		[33]
Municipality's water supply		62 %	9/23 stool: NoV		[34]
Drinking and shower water		44·7 %	8/10 stool: NoV		[35]
Recreational fountain water	Conf.	54 %	22/25 NoV in symptomatic children; 6 16 NoV in asymptomatic children; GI.3	Fountain water: GI.3	[36]

Table 2. (*cont.*)

Food/water	Type of evidence	Attack rate	Laboratory investigation		Ref.
			Human samples	Food/water samples	
Pool water		242 people ill	5/6 stool: GII	pool water: GII	[37]
Drinking water		2860 people	28/70 stool: NoV	4/44 tap water + 4/12 seawater: NoV	[38]
Drinking water		218 people ill	11/31 stool: GI.5	Drinking water: GI.	[39]
Drinking water		Around 80 people ill	3 stool: GI.3 and GII.6	Drinking water: GI.3	[40]
Lake water	CC	~400 people ill	18/38 stool: 17 GI + 1 GII		[41]
Drinking water		31·5 %	3/3 stool: NoV		[42]
Ice cubes + tap water		40·6 %	1/2 stool: NoV		[43]
Raspberries					
Bakery with raspberries	Pres.	30 people ill	5/9 stool: GI	GII.b in raspberries	[44]
Frozen raspberries		OB 1: 450 ill	stool: GII.7		
		OB 2: 70 ill	no stool		[45]
		OB 3: 400 ill	stool: GII.4		
		OB 4: 40 ill	stool: GII.b		
		OB 5: 50 ill	stool: GII.7		
		OB 6: 33 ill			
Frozen raspberries, mixed with fresh cheese	Pres.	27·8 %	5/6 stool: GI.5		[46]
Cake: cream and raspberries		OB 1: 80 %			[47]
Cheesecake with raspberries		OB 2: 90·9 %			
Drinks with raspberries		OB 3: 40·0 %			
Dessert with raspberries		OB 4: 100 %			
Salad	CC	9·3 %	4/13 stool: 1 GII.6 + 2 GI.6 + 1 GI.3		[48]

Conf., Confirmed; detection of causative agent in food/water and stool; CC, outbreaks where a case-control study revealed the most probable causative agent; Pres., outbreaks where the causative agent is 'presumably' indicated but not funded by a case control study, cohort study or was not confirmed; OB, outbreak.

In 2007 all inspectors of the Belgian FASFC, which has responsibility for control of foodstuffs and their raw materials at all stages of the food chain as well as investigation of foods implicated in foodborne outbreaks, followed a mandatory specific training on NoV. Furthermore, since 2006 the medical health inspectors of the Belgian regional communities have had the opportunity to send faecal samples to the National Reference Laboratory for foodborne outbreaks.

Despite the measures taken to improve the investigation regarding NoV outbreaks in Belgium, there was only one outbreak where NoV was detected in both food and stool samples. Underreporting, late reporting, the lack of clinical and environmental samples as well as the lack of laboratories to test water and food for NoV hamper the recognition of the role of this virus in outbreaks [51]. Moreover, the FBVE network has encountered similar problems [3]. The percentage of outbreaks with complete data including epidemiological data and laboratory findings are increasing each year, indicating better cooperation between laboratories and epidemiological units [52].

In 6/10 foodborne outbreaks due to NoV in Belgium, the causative agent, i.e. NoV, was detected

in food samples which is unique. Internationally reported NoV outbreaks mostly include just epidemiological information and/or stool sample analysis. It is rare for detection of NoV in food other than shellfish or water to be described. The foods implicated in the Belgian outbreaks (sandwiches, soup, meat stew, mashed potatoes, a composite meal) were probably contaminated with low levels of NoV on the surface of the food item. Contrary to these composite and complex food matrices, shellfish bioaccumulate and concentrate NoV, facilitating the detection of the virus in this food category [53]. Moreover, the food items cover diverse types of foods and were all tested with the same NoV extraction and detection method. This extraction protocol enabled the isolation of NoV in ham, salami and spare ribs in The Netherlands [11]. Consequently, this method has proved useful in outbreak investigations as suggested by Boxman *et al.* [11].

The Belgian data were compared with 40 international outbreak events between 2000 and 2007. It should be noted that the number of international outbreaks considered are extremely low and probably biased because peer-reviewed publications of food- and waterborne outbreaks report merely large, well documented, unusual or novel events [52, 54].

In the Belgian NoV outbreaks reported, NoV GII was detected in 80% of the NoV outbreaks and NoV GI in the remaining 20%. GII.4 and GII.2 were confirmed in two outbreaks. An attempt was undertaken to sequence other samples as well but was unsuccessful. The time between detection and sequencing (up to more than 1 year) and the low levels of NoV on food are factors that resulted in the failure of obtaining sequencing data from food samples.

The Belgian NoV outbreaks were characterized by an attack rate ranging between 3·0% and 100%, similar to the international data. This broad range of observed attack rates is not surprising because outbreaks are caused by accidental point contamination. Less fluctuating attack rates were noted for shellfish. Raspberries are likely to be contaminated by water in the field, affecting large batches. One batch of raspberries can be used for several desserts on different locations and can therefore cause outbreaks at several settings such as in households, at meetings and parties, nursing homes and schools with a considerable high attack rate. Raspberry outbreaks were recorded in particular in summer and in Nordic countries where desserts based on raspberries are very popular.

The role of the food handler was epidemiologically suggested in 80% of the NoV outbreaks reported in Belgium. This high percentage was found because NoV analysis was considered in outbreaks where foods were prepared by food handlers irrespective of the food type. In 17/40 studied internationally reported outbreak events, the role of the food handler was indicated. In eight of these 17 outbreaks, a sick food handler or food handler with a recent history of gastroenteritis was observed [11, 14, 18–23]. Moreover, the majority of these outbreaks were linked to sandwiches as the contaminated source of the outbreak. Similarly, in two Belgian outbreaks, history of gastroenteritis was reported by persons handling sandwiches. In January and May, a high incidence of food handler-associated outbreaks occurred. This trend was not observed in Belgium because of the limited number of outbreaks.

NoV GII.4 outbreaks and sporadic cases involving food handlers with gastroenteritis in Japan revealed that many asymptomatic food handlers also tested positive for NoV GII.4 strain. Moreover, the number of virus shed by symptomatic and asymptomatic food handlers was similar, indicating the potential hazard of these highly contagious viruses [55]. More patients tend to be involved in outbreaks due to food handlers compared to oyster-related outbreaks because in general large catering establishments are implicated compared to oyster-associated outbreaks [56]. Poor personal hygiene was also identified as a contributory factor in outbreaks where NoV was assigned as the causative agent [51]. Therefore, it is of major importance to inform food handlers of their responsibilities for the prevention of large-scale NoV outbreaks. Food handlers must have knowledge of good hygienic practices. If family members of food handlers or food handlers themselves show symptoms of gastroenteritis, the employer should be alerted in order that the appropriate preventive measures can be taken. AQ7

The present study shows that the introduction of a detection method increased the number of reported NoV outbreaks in Belgium from none in 2005 to becoming the leading cause of foodborne outbreaks in 2007. Food handlers appeared to be the most probable source of foodborne NoV outbreaks and these outbreaks were frequently associated with sandwiches.

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DECLARATION OF INTEREST

AQ8 None.

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Epidemiological study of bovine norovirus infection by RT-PCR and a VLP-based antibody ELISA

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ABSTRACT

Noroviruses, belonging to the family *Caliciviridae*, have been identified in human beings and in several animal species including cattle. The distribution of bovine norovirus infections was investigated by both RT-PCR to detect norovirus genomes and a virus-like particles-based ELISA to detect genotype 2 bovine norovirus antibodies. During a 1-year systematic study, a virus prevalence of 7.5% (CI 95%: [3.7; 13.4%]) (10 out of 133 samples) was found in stool samples from diarrhoeic calves screened by RT-PCR. Nucleotide sequencing performed on the polymerase region classified all the norovirus amplicons in the bovine norovirus genotype 2. Rather surprisingly, some rotavirus sequences were also detected. On the basis of the polymerase region, genotype 1 bovine norovirus was not identified. Other enteropathogens were found in all samples. By ELISA, a genotype 2 seroprevalence of 93.2% (CI 95%: [90.4; 95.3%]) was found from calves and adult cattle. Antibody levels against genotype 2 bovine noroviruses rose in the first 6 months of life and were maintained in adults. Together the results of virus prevalence and seroprevalence studies suggest that bovine norovirus infection occurs early in life and that re-infection with serologically related bovine noroviruses strains could occur in adult cattle as reported for rotaviruses. The antibody rise against genotype 2 bovine noroviruses in the adult cattle also suggests a short lived and/or strain specific immunity as already shown in human noroviruses. Genotype 2 bovine noroviruses are endemic in the region investigated.

1. Introduction

Noroviruses (NoV) belong to the family *Caliciviridae* and have been identified in the human being and in several animal species including the bovine (Scipioni et al., 2008b; Green, 2007). Noroviruses are small, round, non-enveloped viruses, with a 25–40 nm diameter and have a positive-sense, single-stranded RNA genome with three open reading frames (ORF). The non-structural proteins, including the viral RNA-dependent RNA polymerase (RdRp), the single capsid protein (VP1) and the minor structural protein are encoded by ORF1, 2 and 3, respectively (Green, 2007). Ninety dimers of VP1 form the capsid which presents an icosahedral symmetry under electron microscopy (EM). Capsid protein VP1 has a molecular weight of around 55–60 kDa and is organised into two domains: a shell (S) domain (N-terminal part of VP1) and a protruding (P) domain (C-terminal part of VP1) (Prasad et al., 1999). The P domain supports receptor recognition and immunogenic functions (Lochridge et al., 2005). Noroviruses are difficult to grow in cell culture but abundant quantities of virus-like particles (VLP) can be obtained by expressing VP1 in the baculovirus protein expression system. These VLP are morphologically and antigenically similar to the native strain (Jiang et al., 1992).

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Bovine norovirus (BoNoV) prototype strains identified so far are Newbury2 strain (NB2), previously known as Newbury Agent 2, and the Jena virus (JV). The NB2 and JV were identified by EM in the faeces of diarrhoeic calves in 1978 in Great Britain (Woode and Bridger, 1978) and in 1980 in Germany (Gunther and Otto, 1987). They were later genetically identified as belonging to the genus *Norovirus* (Liu et al., 1999; Oliver et al., 2003) where all BoNoV strains fall into the genogroup III (Ando et al., 2000). Two genotypes are described in the BoNoV genogroup: viruses genetically related to JV were assigned to the genotype 1 and viruses genetically related to NB2 to the genotype 2 (Ando et al., 2000).

Their relative environmental stability, their main faecal-oral transmission route, their low infectious dose and their large strain diversity increase the risk of cattle infection with these viruses (Green, 2007). In gnotobiotic calves, BoNoV induce non-hemorrhagic enteritis, mild diarrhoea, transient anorexia and malabsorption (Woode and Bridger, 1978). But the real impact of these viruses in the field is currently poorly evaluated. The two genotypes of BoNoV were recently shown to be endemic in Great Britain and Germany (Oliver et al., 2007b). They were also isolated in all continents and thus seem to be widespread (Scipioni et al., 2008b).

In this work, an original combined virological and serological approach was used to characterise the epidemiology of BoNoV infection in cattle. In a first virological approach, the molecular prevalence was investigated in diarrhoeic calf samples during a 1-year study. In a second serological approach, a VLP-based ELISA was developed and the cattle seroprevalence against BoNoV was estimated in the same region. Together the results suggest an association of BoNoV infections with the age of the animals.

2. Materials and methods

2.1. Bovine stool samples and genetic analysis

Stool samples from calves aged 1 week to 6 months were systematically received from a Belgian diagnostic laboratory each month through the year 2007. Samples came from different places out of five provinces of Belgium (Hainaut, Namur, Liege, Luxembourg, Walloon Brabant). Their origin was either from diarrhoeic samples ($n = 74$) submitted for etiologic diagnosis of gastroenteritis or from necropsy samples ($n = 59$) where gastroenteritis lesions were found. This stool bank ($n = 133$) was screened by one step RT-PCR for the presence of BoNoV genome sequences with Quick Access kit (Promega, Madison, WI, USA). Four different primer pairs were used to amplify segments of the polymerase gene and the beginning of the capsid region: CBECu (Smiley et al., 2003), JV12/13 (Vinje et al., 2003), P289/290 (Jiang et al., 1999) and BEC (Ike et al., 2007). These primers were selected in order to perform the broadest detection of NoV including a possible zoonotic transmission. Out of the BoNoV specific primers (CBECu and BEC), CBECu pair was seen to detect not only genotypes 2 but also genotypes 1 BoNoV with an apparent sensitivity of about 83% relative to the p289/290 pair in a previous study (Smiley et al., 2003). RT-PCR program consisted in a reverse transcription step of 45 min at 45 °C followed by the PCR reaction. It consisted in an initial denaturation step of 2 min at 94 °C followed by 40 PCR cycles (30 s denaturation step at 94 °C; 1 min annealing step at 48 °C for CBECu, JV12/13 and BEC primers, 51 °C for p289/290 primers; 2 min extension step at 68 °C) and a final extension step of 10 min at 68 °C. Positive amplicons were purified from agarose gel and cloned into pGEMt Easy (Promega, Madison, WI, USA). Sequencing reactions were carried out on three clones of each amplicon with the BigDye terminator kit version 3.1 (AppliedBiosystem, Foster City, CA, USA) and resolved with an ABI 3730 automatic capillary sequencer (AppliedBiosystem, Foster City, CA, USA). Nucleotidic sequences were analysed with the BioEdit Sequence Editor version 7.0 software. Nucleotidic similarity with the NCBI genetic database was assessed using the BLAST tool (available at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Phylogenetic inference was performed with the MEGA version 4 software package (Tamura et al., 2007). Phylogenetic tree was constructed by neighbor-joining analysis where evolutionary distances were computed using the Maximum Composite Likelihood method. The confidence values of the internal nodes were calculated by performing 1000 replicates bootstrap values.

2.2. Construction of a recombinant baculovirus for the BoNoV capsid protein

A long fragment product (RdRp region to the poly-A tail) from the genome of a genotype 2 BoNoV strain previously isolated in the laboratory (named B309) was generated by RT-PCR and cloned into pTopoXL (Invitrogen, Carlsbad, CA, USA). The insert was sequenced by primer walking. The sequence coding for the ORF2 was isolated and mutations in this sequence were evaluated with the vector NTI 6 software (InforMax, North Bethesda, MD, USA). Specific forward and reverse PCR primers to amplify the ORF2 were selected using the Vector NTI program (InforMax, North Bethesda, MD, USA). These primers include the EcoRI (B309_EcoRI, aaagaattcaatgaagatgact), and HindIII (B309_HindIII, cccaagctttcagaagccatcaagacggggaaggcg) restriction site upstream and downstream of the start and stop codon of the ORF2, respectively. The PCR product was cloned into the EcoRI/HindIII digested pFastBac Dual plasmid of the bac-to-bac system (Invitrogen, Carlsbad, CA, USA). A recombinant plasmid was used to transform MAX Efficiency DH10bac competent cells (Invitrogen, Carlsbad, CA, USA) where transposition between the recombinant pFastBac Dual and a baculovirus shuttle vector (bacmid) occurred. Recombinant bacmid purification was performed from positive clones with the Plasmid Purification Mega kit (Qiagen, Valencia, CA, USA). The transposition was controlled by PCR with M13 primer pair. Monolayers of *Spodoptera frugiperda* (Sf9) insect cells were grown in Sf-900 II medium (Invitrogen, Carlsbad, CA, USA) supplemented with a 10% association of penicillin (5000 U/ml)–streptomycin (5 mg/ml) (Invitrogen, Carlsbad, CA, USA) and subcultured when they reached

confluence. Transfection of Sf9 cells was conducted in 6-wells according to the manufacturer protocol to generate a recombinant baculovirus for the BoNoV capsid protein.

2.3. Expression and purification of the capsid protein of a bovine norovirus

A recombinant baculovirus stock was generated and monolayers of Sf9 insect cells were infected at a multiplicity of infection of 10. Five days post-infection, cells and supernatants were harvested and submitted to three freezing/thawing cycles. It was followed by low speed centrifugation to remove cell debris and by high speed centrifugation through 5% sucrose cushion in a Beckman SW28 rotor at 100,000 × *g* for 2 h. The pellet was suspended in phosphate buffered saline (PBS) and centrifuged through a 35–65% sucrose gradient at 100,000 × *g* for 18 h in a Beckman SW28 rotor. The gradient was fractionated and each fraction was dialysed separately against PBS in 25 kDa porous Spectra/Por membrane (VWR, Leuven, Belgium). Protein concentration in each gradient fraction was evaluated by BCA (Pierce, Rockford, IL, USA). Fractions were then subjected to verification for the presence of either capsid protein as VLP.

2.4. Validation of the production of the bovine norovirus-like particles

An aliquot of each fraction was analysed by Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) with the NuPAGE kit (Invitrogen, Carlsbad, CA, USA) to select fractions containing proteins at a molecular weight of about 55–60 kDa. These fractions were analysed under 80 kV EM examination (Philips EM208 transmission electron microscope). Prior to the examination, samples were submitted to ultracentrifugation on grids with increased hydrophilicity by Alcian blue pre-treatment and subjected to negative staining by phosphotungstic acid or uranyl acetate.

Serum from a calf experimentally infected with NB2 (Bridger et al., 1984) was used in western blot to detect the B309 BoNoV capsid protein. Briefly, a positive fraction, non-infected Sf9 cells and wild type baculovirus infected Sf9 cells were blotted to a nitrocellulose membrane after electrophoresis under denaturing or non-denaturing conditions. The membrane was blocked overnight at room temperature with PBS complemented with 0.05% tween 20 (PBST 0.05%) and 20% horse serum. The second day, blocking solution was removed and the membrane was incubated for 1 h at room temperature in PBS containing 20% horse serum and the 1000-fold diluted calf serum. After three washes with PBST 0.05%, the membrane was incubated for 1 h in PBS containing 20% horse serum and a horseradish peroxidase (HRP) conjugated, 10,000-fold diluted, anti-bovine immunoglobulins (Ig) G rabbit IgG. After three washes with PBST 0.05%, the chemiluminescent peroxidase substrate (Sigma–Aldrich, Salt Lake city, UT, USA) was added on the membrane and it was revealed in dark room on Kodak BioMax Light film (Sigma–Aldrich, Salt Lake City, UT, USA).

2.5. Serum sampling

Blood samples were taken from 439 calves and adult cattle from different premises in Belgium. Serum samples originated from the same area that stool samples but from different premises. The sera were grouped according to the age of the animals. Seven age classes were determined: 1-week to 1-month old ($n = 20$), 1–6 months old ($n = 29$), 6 months to 1-year old ($n = 76$), 1–3 years old ($n = 105$), 3–6 years old ($n = 123$), 6–9 years old ($n = 59$) and older than 9 years old ($n = 27$). In the addition to the 439 sera, a collection of 30 serum samples of colostrum deprived newborn calves from the same region was used as a negative serum control. A commercial bovine anti-rabbit IgG antibody (Bethyl laboratories, Montgomery, TX, USA), purified by affinity chromatography, was also used to test the specificity of the ELISA. The serum of the NB2 experimentally infected calf P131 was used as a positive control (Bridger et al., 1984).

2.6. Evaluation of the seroprevalence against BoNoV by ELISA

Positive gradient fractions by both SDS-PAGE analysis and EM examination were selected for the ELISA. Ninety-six well Microlon plates (Greiner Bio-one, Piscataway, NJ, USA) were coated with 100 μl of a VLP solution in PBS (2 ng/μl) and incubated 1 h at 37 °C. They were washed once with PBS and then blocked with PBS complemented with 0.1% tween20 (PBST 0.1%) and with a 4% casein hydrolysate (PBST/HC). Plates were washed once with PBS and then incubated with the 100-fold diluted bovine serum samples in triplicate at 37 °C. After 1 h and three washes with PBST 0.1%, 10,000-fold diluted, HRP conjugated rabbit IgG anti-bovine IgG were applied to each well. After incubation at 37 °C for 1 h, wells were washed three times with PBST 0.1% and 50 μl of the substrate of HRP, tetramethylbenzidine (Sigma–Aldrich, Salt Lake city, UT, USA), were added. The reaction was stopped after 5 min by addition to each well of 50 μl of H_2SO_4 2 M. Optical densities (OD) at 450 nm were measured with a Thermo Labsystems Multiskan microplate reader and analysed with Ascent Software for Multiskan version 2.6 (Thermo Labsystems, Waltham, MA, USA). All sera were analysed on the same day. The cut-off value for the indirect ELISA test was obtained from the serum samples of the 30 colostrum deprived calves (mean + 1.96 standard deviation) and the apparent prevalence was determined on the 439 bovine serum samples with a 95% confidence interval assuming a binomial exact distribution. All statistical analyses were carried out with the STATA/SE software (Stata Corp., College Station, TX, USA). The OD means in the different age groups of animals (unequal variance and non-normal distribution) were compared with the Wilcoxon rank-sum test (Dagnelie, 1998). The limit of statistical significance of the conducted test was defined as $P \leq 0.05$.

3. Results

3.1. Molecular prevalence of bovine norovirus infection

A molecular prevalence of 7.5% (CI 95%: [3.7; 13.4%]) for BoNoV infection was found in the stool samples of

diarrhoeic calves. Twelve potentially positive amplicons were generated by use of the different primer pairs: 9 with the CBECu pair, 1 with both the CBECu and the BEC pairs, 1 with both the CBECu and the JV12/13 pairs and 1 only with the BEC pair. No amplicon was generated with the p289/290 pair. By BLAST and phylogenetic analysis, 10 amplified polymerase sequences were genetically related to NoV genogroup III genotype 2 strains (Fig. 1). The two other ones, amplified with the CBECu pair at a lower molecular size than expected (about 418 bp), matched with about 90% identity with bovine rotavirus polymerase (VP1) gene sequence. These two samples were also confirmed rotavirus-positive by ELISA based on the rotavirus VP6 detection.

BoNoV were found equally in diagnostic and necropsy samples. Another etiology (rotavirus, coronavirus, *Cryptosporidium parvum* or *Escherichia coli*) was found in all the 10 BoNoV positive samples by different tests performed in a diagnostic laboratory. Remarkably, the sample BV119 was positive by ELISA for bovine rotavirus but no rotavirus nucleotidic sequence was amplified with the CBECu pair in this sample.

3.2. Virus-like particles of the bovine norovirus isolate B309

The B309 BoNoV isolate was previously related to the genotype 2 BoNoV in its RdRp region. By BLAST after the sequencing of its C-terminal part, the B309 isolate shared 89% of nucleotide and 98% of amino acid identity with the NB2 in the entire capsid gene. The B309_EcoRI/B309_HindIII amplified ORF2 sequence was compared to the original sequence and two mutations were found. The first mutation was a transition in the 5th codon and modified the composition in amino acid ($Asp_5 \rightarrow Asn_5$). The second mutation was located in the 97th codon and was a silent mutation. A recombinant bacmid for the B309 ORF2 was generated and 72 h after recombinant baculovirus infection, proteins of about 57 kDa could be detected by SDS-PAGE analysis in several fractions obtained by the gradient applied on harvested cells and supernatants (Fig. 2). Bioinformatic analysis with the Vector NTI software predicted a similar molecular weight for the B309 capsid protein. Particles from 30 to 40 nm range diameter with typical calicivirus cup-shaped depressions were observed under EM examination of some positive fractions of the gradient (Fig. 3).

3.3. Western blot analysis

By western blot analysis, fractions containing B309 VLP reacted at about 57 kDa with a serum resulting from the immunization of a calf with NB2 strain. A weak positive band was also obtained in the lane containing Sf9 cells infected by a wild type baculovirus (Fig. 4A). Western blot performed under non-denaturing conditions revealed a band at about 120 kDa with VLP but not against mock

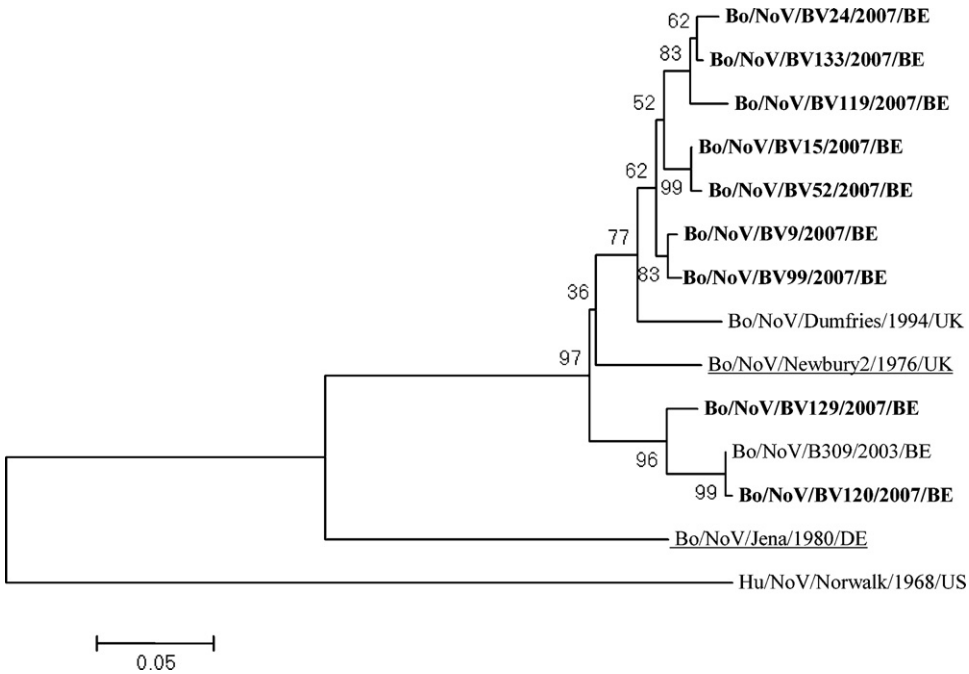

Fig. 1. Neighbor-joining phylogenetic tree based on the RNA-dependent RNA polymerase/capsid protein coding region (429 nt) of bovine noroviruses and the Norwalk virus. Bovine norovirus isolates identified in this study (in bold face) are genetically compared to bovine norovirus reference strains (underlined) and previously isolated strains. Scale bar represents the phylogenetic distances expressed as units of expected nucleotidic substitutions per site. Bootstrap values (1000 replicates) are reported. Genbank accession numbers of strains used: AF097917 (Bo/NV/Newbury2/1976/UK); AJ011099 (Bo/NV/Jena/1980/DE); AY12674 (Bo/NV/Dumfries/94/UK); EU877966 (Bo/NV/BV9/2007/BE); EU877967 (Bo/NV/BV15/2007/BE); EU877969 (Bo/NV/BV24/2007/BE); EU877970 (Bo/NV/BV52/2007/BE); EU877971 (Bo/NV/BV99/2007/BE); EU877972 (Bo/NV/BV119/2007/BE); EU877973 (Bo/NV/BV120/2007/BE); EU877974 (Bo/NV/BV129/2007/BE); EU877975 (Bo/NV/BV133/2007/BE); M87661 (Hu/NV/Norwalk 8FIIa/1968/USA). The tenth amplicon, Bo/NV/BV18/2007/BE, (Genbank accession number EU877968), was not introduced in the tree because its sequence was shorter.

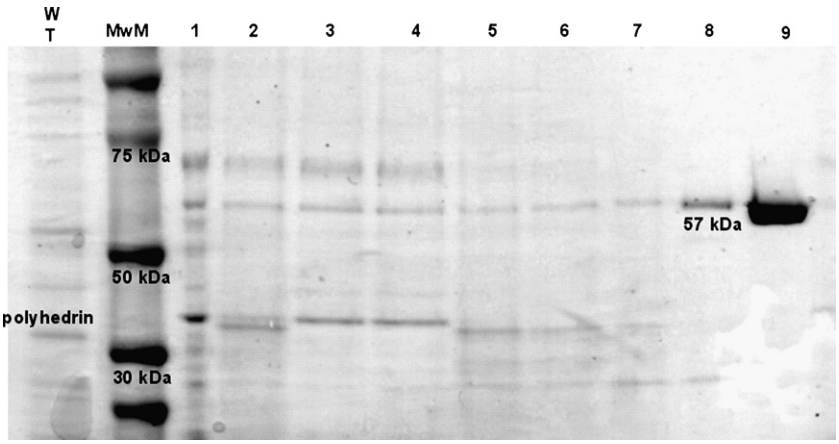

Fig. 2. Supernatants of the baculovirus expression of the capsid protein of the B309 isolate were submitted to purification in sucrose gradient. SDS-PAGE analysis was performed on each fraction and the membrane was stained with Coomassie blue. Results for some fractions are reported here (lanes 1–9). Proteins of about 57 kDa, the expected molecular weight for the B309 capsid protein, were shown. Supernatant of Sf9 cells infected by a wild type baculovirus (lane WT) was used as negative control. MwM: molecular weight marker.

infected or Sf9 cell components recovered after wild type baculovirus infection (Fig. 4B).

3.4. Seroprevalence against BoNoV genotype 2 in the Belgian cattle

Only fractions positive to EM examination (visualization of VLP) were used in the ELISA format. Analysis of sera from colostrum deprived newborn calves allowed the definition of a cut-off value of 0.3 for the ELISA. Optical density values obtained for the bovine anti-rabbit IgG serum (mean: 0.1) were similar to those of colostrum deprived newborn calves. By statistical analysis, a high seroprevalence of 93.2% (CI 95%: [90.4; 95.3%]) was found in Belgian cattle sera. Significant statistical differences were found between the mean OD value of different age groups of animals: between the colostrum deprived calves and the 1-week to 1-month-old calf groups, the 1–6 months old and 6 months to 1-year-old groups, and the 6 months to 1-year-old group and the 1–3 years old group. In the boxplot graph, median value of the "1 week to 1 month old" class rose dramatically compared to the median value of the newborn class. This value was maintained during the "1 month to 6 months old" period and was followed by a new rise in the "6 months to 1 year old" age class. A drop in the values of medians was shown between this last class and the "1 to 3 year old" class. Medians were maintained thereafter in the adult period (Fig. 5). Remarkably, during this period, some OD values were below the cut-off (dots below the line at 0.3 OD value in Fig. 5) and were similar to the values recorded during the colostral period.

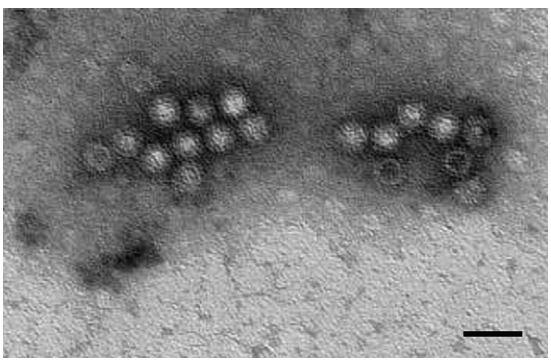

Fig. 3. Electron micrographs of virus-like particles (VLP) of the bovine norovirus B309 isolate. The VLP were produced by expressing the capsid protein in the baculovirus protein expression system and purified in a sucrose gradient. The photograph was taken from the fourth fraction. After uranyl acetate negative staining on Alcyan blue treated greads, particles with the specific size (approximately 40 nm) and morphology of calicivirus-like virions were readily observed. The nucleocapsids were isometric, showed icosahedral symmetry and the border has cup-shaped depressions. Bar, 50 nm.

4. Discussion

Although HuNoV are known to be widely distributed and already recognized as a threat to public health, the impact of BoNoV is still poorly studied. In Europe, very few countries have performed epidemiological studies. The 1-year prospective study reported in the present paper endorsed that genotype 2 BoNoV are endemic in European cattle. A BoNoV prevalence of 7.5% was found in stool samples of diarrhoeic calves. In the serological approach, a strong seroprevalence (93.2%) against genotype 2 BoNoV was determined with a VLP-based antibody ELISA in cattle of the same region. Together, the results of prevalence and seroprevalence allowed the description of the evolution of the antibody levels against genotype 2 BoNoV infection during the life of the cattle.

The molecular prevalence of BoNoV in samples coming from the Belgian diagnostic laboratory was in accordance with studies in Great Britain (Milnes et al., 2007) and Korea where Park et al. (2007) report also a majority of genotype 2 infection. The genotype of the strains detected in the British study was not clarified. The prevalence determined by RT-PCR analysis in this study is also similar to the results obtained by an antigen capture ELISA in Germany and developed using a genotype 1 JV strain (Deng et al., 2003). As genotypes 1 and 2 BoNoV are antigenically

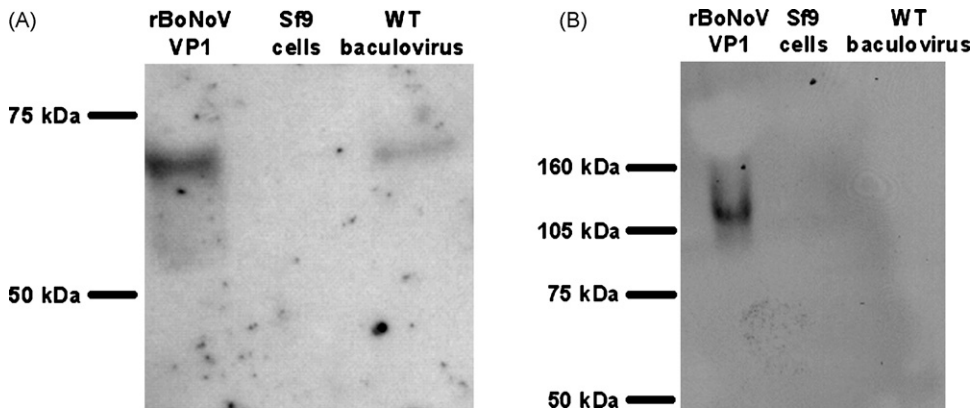

Fig. 4. Western blotting of the expressed capsid protein of B309, mock infected Sf9 cells and wild type baculovirus infected Sf9 cells. (A) After electrophoresis under denaturing conditions, a fraction of the applied gradient reacted at about 57 kDa with the serum of a calf experimentally infected with Newbury2 strain, a genotype 2 genogroup III norovirus, but also against Sf9 cells infected by wild type baculovirus. (B) After electrophoresis under non-denaturing conditions, a reaction was obtained at about 120 kDa only against the fraction resulting from purification.

distinct (Oliver et al., 2006), the true BoNoV prevalence could be higher or a preferential geographic distribution could exist. In contrast, Mattison et al. (2007) detected only 1.6% positive in stool samples from a random study in Canadian cattle but Smiley et al. (2003) detected up to 72% of samples positive in two farms in USA. In a longitudinal study in The Netherlands, prevalences of 31.6 and 4.2% were found in farm stool samples and individual samples of dairy cattle, respectively (Van Der Poel et al., 2003). Thus, prevalence results by RT-PCR seem to depend on the continent, the country, the sampling strategy and especially on the test conditions (e.g. annealing temperature, primers, use of internal control as mentioned in Scipioni et al., 2008a). Also the type of study, cross-sectional versus longitudinal as a part of the Dutch study, could explain some differences. BoNoV infection seems to be strongly related to the age with a higher rate in the first year of the animal life. Positive results were obtained along all the sampling period (data not shown) but further research is needed to investigate a potential seasonality for the infection unlike as described for HuNoV (Mounts et al., 2000). In our results, we detected more isolates with the CBECu pair than with all the other pairs. Also, the CBECu primers allowed the detection of two bovine rotavirus

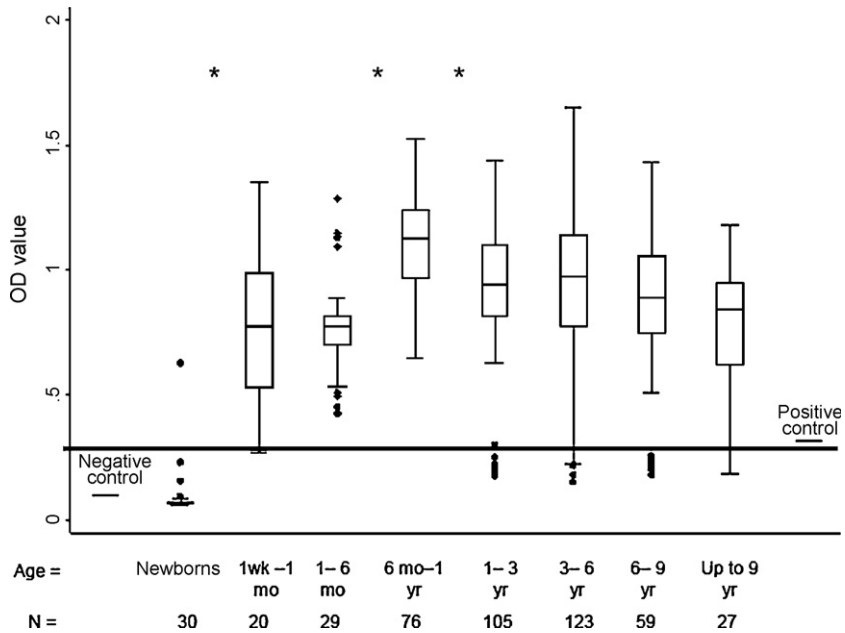

Fig. 5. Seroprevalence against bovine noroviruses in the Belgian cattle. The OD means of the different age groups of animals, collected in the ELISA format (unequal variance and non-normal distribution), were compared with the Wilcoxon rank-sum test. The distribution of the optical densities obtained with the indirect ELISA is represented in a box plot format where optical densities are reported in function of age classes. An asterisk highlights significant statistical differences between means of the different age groups. The cut-off is represented by the line at 0.3 and the dots represent extreme values. OD: optical density, negative control: optical density value obtained for the bovine negative serum (anti-rabbit IgG), positive control: optical density value obtained for the positive control (calf experimentally infected with the Newbury2 strain, Bridger et al., 1984), mo: month, wk: week, yr: year.

infections with however a small difference in the molecular weight for the amplicon (418 bp versus 532 bp). To our knowledge, such observation has never been reported and it could account for some differences between RT-PCR prevalence results depending on the primers used. Nucleotide similarities were found between the CBECu primers and the prototype bovine rotavirus sequence to which they matched by BLAST. On the 23 nt of the CBECu-F sequence, 14 nt including the last five nt matched exactly with the sequence of the bovine rotavirus polymerase (VP1, genbank accession number: J04346). Nucleotide similarity for CBECu-R was smaller. The CBECu-F primer was designed to match the widely conserved YGDD motif in RNA-dependent RNA polymerases of RNA viruses (Bruenn, 1991) and this could explain the detection of bovine rotavirus. Some strains were detected with primers designed to detect HuNoV (JV pair) but at a low rate, as previously reported (Van Der Poel et al., 2003). No HuNoV sequences were identified despite the use of two primer sets designed for their detection, suggesting at least a very low prevalence of these human viruses in cattle. Despite the variety of the primers used in the present study, the lack of detection of the genotype 1 BoNoV could be explained by suboptimal primer use for the RT-PCR (Oliver et al., 2007b). The age of the cattle population studied was also suggested as an explanation for the lower detection of genogroup III genotype 1 NoV (Oliver et al., 2007b). As RT-PCR primers used in this study only target the viral polymerase region and thus miss potential recombinant strains, the possibility that some positive samples contained chimaeric bovine noroviruses could be another explanation for the low apparent prevalence of GIII.1 noroviruses.

Interestingly, few amino acid mutations were detected between the native B309 strain sequence (year of isolation: 2003) and the NB2 strain sequence (year of isolation: 1976) (Scipioni et al., submitted for publication). Nearly all mutations were located in the P domain with surprisingly relatively few mutations in the P2 sub-domain, the most variable region of VP1 known to support antigenic determinants and drift-induced antigenic variation in NoV strains (Allen et al., 2008; Lochridge and Hardy, 2007).

The two mutations detected in the VLP constructed in the baculovirus system compared to the native B309 strain, did not affect the antigenicity by their effect and their localisation in the amino acid sequence. Oliver et al. (2007a) reported that the P131 calf serum did not react in western blotting with BoNoV genotype 2 VLP in denaturing reaction but react at a higher molecular weight than expected under non-denaturing conditions. In our conditions this serum reacts against a fraction containing VLP but also with infected Sf9 cells, in denaturing conditions. To test the specificity of this reaction against infected Sf9 cells, western blots were carried out with foetal calf serum and serum from a colostrum deprived newborn calf used in this study. With these two sera, a positive band at the same molecular weight was observed with baculovirus infected Sf9 cells under denaturing conditions whereas no reaction was detected in non-denaturing conditions (Mauroy and Thiry, unpublished results). These data confirm the non-specific binding of bovine immunoglobulins on denatured protein extracts from baculovirus infected Sf9 cells. In non-denaturing conditions, the serum only reacts against VLP. Thus, B309 VLP are representative of the genotype 2 BoNoV.

A high seroprevalence (93.2%) against genotype 2 BoNoV was found by ELISA. Genotypes 1 and 2 in the BoNoV genogroup, although antigenically distinct, were shown to cross-react in ELISA to the heterologous VLP but at low serum dilution ($\log_{10}$ 1.7) (Oliver et al., 2006). However the true genotype 2 seroprevalence should still remain high, even corrected for a lower sensitivity and specificity of the test. The OD values obtained with the serum raised against rabbit IgG in bovine confirmed the selected cut-off. Almost all colostrum deprived calves did not have antibodies against BoNoV as previously described (Deng et al., 2003). It suggests that BoNoV infection is mainly localised in the intestine, according to the observations with HuNoV (Mattner et al., 2006), and that vertical transmission of the virus is rare. The rise in antibody levels in 1 or 2 weeks old calves suggests an acquisition of antibodies through colostrum absorption or from early infection. Information on prevalence brought by the RT-PCR analysis suggests that the risk of infection during the first 6 months of life is high. The antibody levels determined by ELISA rise again in the post-colostral period (6 months to 1-year old). These results could be explained by a large exposure during this period and the development of natural immunity.

The finding that cattle aged 3–9 years still had antibodies suggests either a long lasting immunity against BoNoV or frequent re-infections. Several characteristics of NoV (environmental resistance, fecal-oral transmission) and farming practices in developed countries could increase the risk of re-infection in cattle, whereas such re-infection was not observed during a prospective RT-PCR study in The Netherlands (Van Der Poel et al., 2003) and during a cross-sectional RT-PCR study performed by ourselves on stool from up to 6 months old cattle ($n = 47$) housed in two different premises (Mauroy and Thiry, unpublished results). However in a retrospective study in Great Britain, Milnes et al. (2007) have amplified norovirus sequences by RT-PCR in adult stool samples. The detection of high levels of antibodies in the adult also suggests their presence in the colostrum. It could explain the antibody rise in neonates after colostrum intake. Remarkably, in contrast to the 1-week to 1-year-old group, some OD values of adult cattle were below the cut-off of the test. These data suggest that antibody levels decrease in some adult cattle reinforcing the hypothesis of a short lived immunity.

The zoonotic risk associated with animal NoV is widely discussed but is hypothetical. However, several recent data showed that HuNoV replication occurred experimentally in gnotobiotic animals including calves (Souza et al., 2008). Furthermore, Mattison et al. (2007) have identified HuNoV sequences in swine and cattle. Shellfish like oysters are known as a main route of food-associated HuNoV outbreaks. As they bind specifically HuNoV to their digestive tissues (Le Guyader et al., 2006) and as BoNoV sequences were also detected in these molluscs (Costantini et al., 2006), new recombinant strains could appear through

mixed infection. Such event was already reported with human strains (Symes et al., 2007). Mixed infections could occur in countries with high population densities of human and production animals and with high prevalence of BoNoV in premises. However, this study did not detect any contaminations of cattle with HuNoV.

Experimental infection of calves with BoNoV has shown their low pathogenicity (Bridger et al., 1984) but their impact in association with classic enteric pathogens in superinfections and in economic losses (weight gain, health status, veterinary treatment cost) has not been studied. In this study, BoNoV were found both in diagnostic samples and necropsy samples and always in association with other pathogens. The role of BoNoV as pathogens in the bovine species should be considered, taking into account their endemicity in the investigated situation and their presence in diarrhoeic calves.

More specific primers, primers targeting both the ORF1-end and the ORF2 regions, a broader sampling strategy, in particular regarding the age of the animals, and an ELISA including VLP of the genotype 1 could refine epidemiological data for BoNoV infection. An Ig discriminative ELISA format (IgA, IgM, IgG1 and 2) could define the status of the animals during their first year of life.

Together, the results of prevalence and seroprevalence show the endemicity of the genotype 2 BoNoV in the studied region. The first 1-year period of life is critical for BoNoV infection. During this period, the immunological status of cattle against BoNoV varies both by colostrum intake and by natural infection.

Conflict of interest statement

The authors ensure that there are no conflict of interest with any person or organisation.

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